A Study of Expression and Function of SepL, a Regulator of Type 3 Secretion in Enterohaemorrhagic *Escherichia coli* O157

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DECLARATION

I declare that all work included in this thesis is my own, except where otherwise stated (Fig.3.12-page 119). No part of this work has been, or will be submitted, for any other degree or qualification.

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ABSTRACT

Enterohaemorrhagic *Escherichia coli* (EHEC) are a recently emerged group of pathogens that can cause fatal infections in the young and elderly. EHEC utilize a virulence factor delivery organelle called a 'Type 3 secretion system' that results in the formation of characteristic 'pedestal structures' on epithelial cells allowing colonization in the human or ruminant gastrointestinal tract. To achieve this, effector proteins have to be injected into host cells. The SepL-SepD complex has been shown to be key for controlling T3-related protein secretion in EHEC. Lack of either protein results in effector hypersecretion and strongly impaired secretion of EspADB translocon proteins. Therefore, the expression and function of SepL was the focus of my PhD research. The expression of SepL was shown to be heterogeneous and co-expressed with EspA filaments in EHEC O157 strains. My work revealed two transcriptional regulators (Ler and SepD) and two putative posttranscriptional regulators (Hfq and CsrA) of SepL expression. Further experiments mapped a key mRNA region required for heterogeneous expression of SepL. This sequence forms a predicted hairpin structure around the Shine-Dalgarno (SD) site of *sepL*. A model has been formed based on my data in which Hfq and CsrABCD bind to the mRNA potentially competing to control translation. Functionally, the C-terminus of SepL was found to be expendable for 1) SepD binding; 2) SepL membrane localization and 3) translocon export, however it was required for 1) limiting effector secretion via (2) a Tir interaction which might be disassociated by (3) an EscD interaction once host cell signals are sensed. Previously, the concept of two different types of T3 secretion signal were demonstrated in *Yersinia* spp, I tested this hypothesis in EHEC using both wild type and SepL/SepD deficient EHEC strains. SepL/SepD is required for the N-terminal signal pathway but not a chaperone binding domain signal pathway. A 12aa NleA which only contained an N-terminal signal was shown to bind to SepD and so did the multi-functional T3 chaperone — CesT. Finally, Far-Western assays demonstrated that SepL only interacted with Tir while SepD could bind other effector proteins indicating that SepL/SepD may act as a targeting hub for effector protein secretion.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABC	**ATP-binding cassette**
AEEC	**Attaching and Effacing *Escherichia coli***
Amp	**Ampicillin**
APEC	**Avian pathogenic *Escherichia coli***
BBB	**Blood brain barrier**
Bp	**Base pair**
BSA	**Bovine serum albumin**
Cam	**Chloramphenicol**
CBD	**Chaperone binding domain**
CDS	**Coding sequence**
Ces	**Chaperone of *Escherichia coli* secretion**
CFP-10	**The 10 kDa culture filtrate protein**
Cif	**Cycle inhibiting factor**
CNF1	**Cytotoxic necrotizing factor type 1**
CR	***Citrobacter rodentium***
Csr	**Carbon storage regulator**
CU	**Chaperone/Usher**
DAEC	**Diffusely adherent *Escherichia coli***
DNA	**Deoxyribonucleic acid**
EAggEC	**Enteroaggregative *Escherichia coli***
EBL	**Embryonic bovine lung**
ECL	**Enhanced chemi-luminescence**
EHEC	**Enterohaemorrhagic *Escherichia coli***
EIEC	**Enteroinvasive *Escherichia coli***
EM	**Electron microscopy**
EPEC	**Enteropathogenic *Escherichia coli***
ESAT-6	**The 6 kDa early secreted antigenic target**
Esc	***Escherichia* secretion**
Esp	***Escherichia* secreted protein**
ETEC	**Enterotoxigenic *Escherichia coli***
ExPEC	**Extraintestinal Pathogenic *Escherichia coli***
FAS	**Fluorescent-actin staining**
FITC	**Fluorescein isothiocyanate**
Gb3	**Globotriaosylceramide**
Gb4	**Globotetraosylceramide**
GFP	**Green fluorescence protein**
GrlA/R	**Global regulator of LEE-activator/repressor**
HC	**Haemorrhagic colitis**
Hfq	**Host factor required for phage Qβ RNA replication**
H-NS	**Histone-like nucleoid-structuring protein**

HlyA	**Alpha-hemolysin**
HM	**Host membrane**
hr	**Hour**
Hrc	**Hypersensitive response conserved**
Hrp	**Hypersensitive response and pathogenicity**
HUS	**Haemolytic uremic syndrome**
IM	**Inner membrane**
Inv	**Invasion (*Salmonella* SPI-1)**
Ipa	**Invasion plasmid antigen**
IRTKS	**Insulin receptor tyrosine kinase substrate**
Kan	**Kanamycin**
Kb	**Kilo base pair**
KDa	**Kilo Dalton**
Lcr	**low-calcium response**
LEE	**Locus of enterocyte effacement**
Ler	**LEE-encoded regulator**
LPS	**Lipopolysaccharide**
LT	**Heat labile toxin**
Map	**Mitochondria associated protein**
MFP	**Membrane fusion protein**
min	**Minute**
MM	**Mycomembrane**
Mpc	**Multiple point controller**
MPEC	**Mammary pathogenic *Escherichia coli***
Mxi	**Membrane expression of Ipa**
NC	**Needle complex**
Nck	**Non-catalytic region of tyrosine kinase adaptor protein 1**
NMEC	**Neonatal meningitis associated *Escherichia coli***
NSS	**N-terminal secretion signal**
N-WASP	**Neuronal Wiskott-Aldrich Syndrome protein**
RNA	**Ribonucleic acid**
mRNA	**Messenger Ribonucleic acid**
ncRNA	**Non-coding Ribonucleic acid**
sRNA	**Small Ribonucleic acid**
rpm	**Revolution(s) Per Minute**
Rsm	**Repressor of secondary metabolites**
Nle	**Non-LEE-Encoded**
OM	**Outer membrane**
Omp	**Outer membrane protein**
ORF	**Open reading frame**
Osp	**Outer *Shigella* protein**
PAI	**Pathogenicity island**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
Per	**Plasmid-encoded regulator**
PMF	**Proton motive force**
Pop	***Pseudomonas* outer protein**
Prg	**PhoP-repressed gene**
QS	**Quorum sensing**
RBC	**Red blood cell**
RFP	**Red fluorescence protein**
rfu	**Relative fluorescence units**

SD sequence	**Shine-Dalgarno sequence**
Sep	**Secretion of *Escherichia coli* proteins**
Sec	**Secretory system**
Sic	***Salmonella* invasion chaperone**
Sip	***Salmonella* invasion protein**
SLT	**Shiga-like toxin**
Spa	**Surface presentation of antigen**
Ssa	**Secretion system apparatus (*Salmonella* SPI-2)**
ST	**Heat stable toxin**
Stx	**Shiga toxin**
STEC	**Shiga toxin producing *Escherichia coli***
T1/2/3/4/5/6/7SS	**Type 1/2/3/4/5/6/7 secretion system**
F-T3SS	**Flagellar T3SS**
NF-T3SS	**Non flagellar T3SS**
Tat	**Twin-arginine translocation**
Tccp	**Tir-cytoskeleton coupling protein**
Tir	**Translocated intimin receptor**
TRITC	**Rhodamine**
Tye	**Translocation of Yops into eukaryotic cells**
UTR	**Untranslated region**
UPEC	**Uropathogenic *Escherichia coli***
VT	**Verocytotoxin**
VTEC	**Verocytoxin producing *E.coli***
Yop	***Yersinia* outer protein**
Ysc	***Yersina* secretion**

PUBLICATION

Chapter 3 was submitted for publication:

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Hierarchal type III secretion of translocators and effectors from *Escherichia coli* O157:H7 requires the carboxy terminus of SepL that binds to Tir. *Mol Microbiol*.;69(6):1499-512. Epub 2008 Jul 30.

Chapter 1

Introduction

1.1 Escherichia coli

Escherichia coli (*Escherichia coli*) is a common bacterial species isolated from the gastrointestinal tract of humans and other mammals. It is named after Theodor Escherich, a German paediatrician who first isolated and studied the organism in 1885 (Escherich, 1885). For a long time *Escherichia coli* was considered a harmless part of the flora present in the lower intestine of humans and warm-blooded animals (Conway, 1995). *Escherichia coli* strains can be benign commensals which benefit their hosts by producing vitamin K2 (Marley *et al.*, 1986) or by preventing the colonisation of pathogenic bacteria within the intestine (Gamage *et al.*, 2006, Leatham *et al.*, 2009). *Escherichia coli* was also adapted as a useful tool for biological research (Lederberg, 1996, Lederberg, 1946, Russo, 2003) and biotechnology (Lee, 1996). However, it was later shown that these 'commensal' *Escherichia coli*, found within gastrointestinal tract or other locations (such as bloodstream, urinary tract, abdominal cavity or gallbladder), could be associated with disease (Johnson and Nolan, 2009, Dho-Moulin and Fairbrother, 1999, Dobrindt, 2005, Dobrindt and Hacker, 2008, Johnson and Russo, 2002, Kaper *et al.*, 2004, Russo and Johnson, 2006, Smith *et al.*, 2007, Vimr and Steenbergen, 2006, Sivick and Mobley, 2010). A further issue is that many strains of *Escherichia coli* are resistant to multiple antibiotics and this may be due to their presence in complex polymicrobial environments like the gastrointestinal tract and their capacity to form biofilms; both factors facilitating the exchange of genetic information (Literak *et al.*, 2009, Pallecchi *et al.*, 2010, Skurnik *et al.*, 2009, Salyers *et al.*, 2004).

1.2 Pathogenic Escherichia coli

Escherichia coli is one species within the large bacterial family, the Enterobacteriaceae, the enteric bacteria, which are facultative anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. Commensal (non-pathogenic) *Escherichia coli* are usually harmless and may actually help the host by preventing pathogen colonization (Gamage *et al.*, 2006, Leatham *et al.*, 2009). However, these commensal strains can be associated with opportunistic infections, for example peritonitis, an inflammation of the peritoneum which can occur following perforation of gastrointestinal tract as a result of injuries or surgery (Levine, 1984). It is now clear that certain *Escherichia coli* strains are more likely to be associated with both intestinal and extra-intestinal infections (Bauchart *et al.*, 2010, Croxen and Finlay, 2010, Johnson *et al.*, 2010, Moriel *et al.*, 2010, Sheldon *et al.*, 2010, Wieser *et al.*, 2010). For example, *Escherichia coli* is a common Gram-negative bacterium isolated from septicaemia cases, especially from patients with compromised immune systems (Orrett and Changoor, 2007) and is one of the most common organisms associated with uncomplicated urinary tract infections (Johnson, 1991). These pathogenic *Escherichia coli* strains are generally separated from other commensal isolates according to the serotype. Serotyping is important to help distinguish the limited number of strains that actually cause disease. In the 1940's Kauffmann turned his attention to the *Escherichia coli* group. He based the serotyping scheme on three types of antigen: the somatic 'O' antigen (O-specific polysaccharide side chain of lipopolysaccharide-LPS), the capsular 'K' antigen and the flagellar 'H' antigen. The K antigens were subdivided into three types depending on their heat-sensitivity. Initially there were 25 O, 55 K and 20 H antigens described (Kauffmann, 1944, Kauffmann,

1947). As new antigens were described new numbers were added to the scheme. Occasionally a number was removed either due to the type strain being reclassified as not being an *Escherichia coli* or the antigen being too similar to another to warrant being given a separate number. More recently, the number of O antigens had reached 173, with 103 K antigens and 56 H antigens (Orskov and Orskov, 1990, Orskov and Orskov, 1992).

1.2.1 Extraintestinal pathogenic *Escherichia coli* (ExPEC)

Escherichia coli acquire sets of genes by horizontal transfer, for example mediated by plasmids and bacteriophages. ExPEC have been shown to share sets of horizontally-acquired virulence determinants as 'pathogenicity islands' (Johnson *et al.*, 2003, Johnson *et al.*, 2010, Johnson and Nolan, 2009, Smith *et al.*, 2007, Johnson and Russo, 2005). There are four main *Escherichia coli* groups involved in extraintestinal infections and these are described below:

1. Uropathogenic *Escherichia coli* (UPEC): *Escherichia coli* is the most commonly isolated bacterial species associated with urinary tract infections (UTI) including cystitis and pyelonephritis (Watts and Hunstad, 2008, Dhakal *et al.*, 2008, Mulvey *et al.*, 2000, Seed and Hultgren, 2005, Wiles *et al.*, 2008, Sivick and Mobley, 2010). 75% of clinical isolates belong to six O-serogroups suggesting that UPEC are a true pathogen group (Johnson, 1991). Important virulence factors associated with UPEC strains include: various filamentous adhesive organelles involved in colonisation (Chen *et al.*, 2009, Cosar *et al.*, 2001, Justice *et al.*, 2006, Lane and Mobley, 2007, Lane *et al.*, 2007, Ong *et al.*, 2008, Rodriguez-Pastrana *et al.*, 2007, Simms and Mobley, 2008b, Simms and Mobley, 2008a, Ulett *et al.*, 2007); the pore-

forming toxin alpha-hemolysin (HlyA) and cytotoxic necrotizing factor type 1 (CNF1) which both contribute to the symptoms of cystitis caused by UPEC infection in humans (Wullt *et al.*, 2000, Miyazaki *et al.*, 2002, Reigstad *et al.*, 2007, Smith *et al.*, 2008).

2. Neonatal meningitis associated *Escherichia coli* (NMEC): While a very rare infection, specific *Escherichia coli* strains can infect babies during the passage through the birth canal (Krohn *et al.*, 1997) and these strains probably invade through the naïve gastrointesinal tract and cause bacteraemia. Once in the blood, NMEC penetrate the blood brain barrier (BBB) causing meningitis. It has been shown that successful crossing of the BBB by *Escherichia coli* requires a high-degree of bacteraemia both *in vitro* and *in vivo* (Kim, 2002, Xie *et al.*, 2004). Meningitis associated with *Escherichia coli* can also occur in individuals with suppressed immune systems (Bonacorsi and Bingen, 2005, Orskov and Orskov, 1985). Interestingly, the majority of *Escherichia coli* isolates from meningitis infection produce the K1 capsule, contributing to survival in the bloodstream as a result of the antiphagocytic properties of the capsule (Wieser *et al.*, 2010, Hacker *et al.*, 1993, Vimr and Steenbergen, 2006);

3. Avian pathogenic *Escherichia coli* (APEC): In most cases, these pathogenic *Escherichia coli* cause colisepticaemia in birds resulting in significant economic losses in the poultry industry. The strains are considered to enter the respiratory tract and colonise the air sacs and then invade the bloodstream (Bauchart *et al.,* 2010, Johnson *et al.,* 2010, Stathopoulos *et al.*, 1999, Dho-Moulin and Fairbrother, 1999, Dozois *et al.*, 2000). Related strains are associated with a number of complex brain, lung and urinary tract diseases in humans and other mammals.

4. Mammary pathogenic *Escherichia coli* (MPEC) — MPEC are a putative group of *Escherichia coli* pathogens causing mastitis in farm animals which has not been well characterised (Shpigel *et al.*, 2008).

1.2.2 Intestinal pathogenic *Escherichia coli*

Escherichia coli normally colonize human gastrointestinal tract immediately (a few hours to days) after birth (Adlerberth *et al.*, 1991, Wold and Adlerberth, 2000). Although most of *Escherichia coli* are harmless, certain serotypes were found responsible for causing diarrhoea in human and animals (Kaper *et al.*, 2004, Hedberg *et al.*, 1997, Hill *et al.*, 1991, Huppertz *et al.*, 1996, Koutkia *et al.*, 1997, Levine, 1987, Levine *et al.*, 1978, Levine and Edelman, 1984, Nagy and Fekete, 1999, Nataro and Kaper, 1998). Here, *Escherichia coli* are classified into 6 groups associated with diarrhoea. These categories are based on differences in virulence properties, adherence patterns and interactions with the intestinal mucosa, O & H types and involvement in distinct clinical syndromes. These groups are:

1. Enteroinvasive *Escherichia coli* (EIEC): these strains produce dysentery that is clinically indistinguishable from shigellosis. It was found that *Escherichia coli* strains isolated from patients with dysentery were also able to cause experimental keratoconjunctivitis in guinea pigs and caused a *Shigella*-like illness in children and adults (Parsot, 2005).

2. Enteroaggregative *Escherichia coli* (EAggEC): these strains are associated with persistent diarrhoea in young children. The distinguishing feature of EAggEC strains is their ability to attach to tissue culture cells in an aggregative manner (Navaneethan and Giannella, 2008).

3. Enterotoxigenic *Escherichia coli* (ETEC): produce diarrhoea resembling cholera but much milder in degree (travellers diarrhoea) (DuPont, 2009). Two types of toxins are produced. Two toxins with related mechanisms of action: (A) Heat labile toxins (LT) which are similar to choleragen. LTs raise adenylate cyclase activity in cells; the subsequent production of cyclic AMP leads to dysregulation of water and ion secretion; (B) Heat stable toxin (ST): these toxins activate guanylate cyclase which also leads to dysregulation of water and ion transport across epithelial cells leading to diarrhoea (Okoh and Osode, 2008, Fleckenstein *et al.*, 2010);

4. Diffusely adherent *Escherichia coli* (DAEC): this is a relatively poorly defined group of strains with some strains considered to contain similar chromosomal regions to the EPEC/EHEC groups described below. (Beinke *et al.*, 1998).

5. Enteropathogenic *Escherichia coli* (EPEC): these strains are often associated with infant diarrhoea. Clinical symptoms include fever, diarrhoea, vomiting and nausea usually with non-bloody stools (DuPont, 2009). EPEC induce a characteristic morphological (A/E) lesion with destruction of microvilli without invasion of the organism (Cleary *et al.*, 2004, Marches *et al.*, 2003, Gauthier *et al.*, 2003a, DeVinney *et al.*, 1999, Rosa *et al.*, 1998);

6. Enterohaemorrhagic *Escherichia coli* (EHEC): these strains cause a diarrhoeal syndrome distinct from EIEC (and *Shigella*) in that there is copious bloody discharge and no fever. Production of Verotoxin or Shiga toxins (also sometimes referred to as "Shiga-like") is highly associated with this group of organisms. These infections can be life-threatening due to effects on vascular endothelium, in particular in the kidneys (haemolytic uremia) (Roe *et al.*, 2003a, Marches *et al.*, 2003, DeVinney *et al.*, 2001, Stordeur *et al.*, 2000, Frankel *et al.*, 1998).

1.2.2.1 Escherichia coli and Shigella

Shigella was originally described by Kiyoshi Shiga, a Japanese physician and bacteriologist, during a severe epidemic with 30% mortality rate in 1897 (Shiga, 1898). *Shigella* is a group of Gram-negative bacteria which are very closely related to *Escherichia coli*. To date, *Shigella* strains have only been associated with dysentery in primates, (Ryan KJ, 2004, Ogawa *et al.*, 2008, Schroeder and Hilbi, 2008). *Shigella* species are indistinguishable from *Escherichia coli* species at the DNA level (Brenner *et al.*, 1969, Hartl and Dykhuizen, 1984). Originally *Shigella* was grouped as a pathogen *Bacillus* to be separated from harmless *Bacillus coli* (*Escherichia coli*) in the early 1940's (Lan and Reeves, 2002). Later, various pathogenic *Escherichia coli* were reported after the first pathogenic *Escherichia coli* — EIEC O124 was described in 1944 (Lan *et al.*, 2004). *Shigella* was reported to share virulence factors with pathogenic *Escherichia coli* (Parsot, 2005, Maurelli *et al.*, 1998, O'Brien *et al.*, 1982, Hartl and Dykhuizen, 1984, Levine, 1987, Jarvis *et al.*, 1995). It was proposed that *Shigella* and pathogenic *Escherichia coli* evolved from non-pathogenic commensal *Escherichia coli* (Levine and Edelman, 1984, Hartl and Dykhuizen, 1984, Kaper *et al.*, 1997, Maurelli *et al.*, 1998, Lan and Reeves, 2002, Fukiya *et al.*, 2004, Lan *et al.*, 2004, Parsot, 2005). *Shigella*, unlike *Escherichia coli*, are lysine decarboxylase positive and do not produce gas when they ferment glucose (Sansonetti, 1999, Maurelli *et al.*, 1998). In addition, *Shigella* strains are non-motile and generally do not ferment lactose whilst most *Escherichia coli* strains are motile and ferment lactose (Sansonetti, 1999, Maurelli *et al.*, 1998).

1.3 Attaching and effacing Escherichia coli (AEEC)

Moon *et al.* (1983) coined the term ""Attaching and effacing" (A/E) and linked this phenotype and EPEC infection together for the first time. This lesion is remarkably different from lesions caused by non-A/E lesion forming pathogens such as ETEC or *V. cholerae*. An A/E lesion is characterised by degeneration of microvilli on gut enterocytes, gross cytoskeletal reorganization of intestinal epithelial cells and intimate attachment between bacteria and host cells (Baldini *et al.*, 1983a, Baldini *et al.*, 1983b, Moon *et al.*, 1983, Knutton *et al.*, 1989a). A/E lesion forming bacteria can be observed embedded on epithelial cells or forming pedestal-like structures on the cell surface (Fig.1.1) (Knutton *et al.*, 1989a, Knutton *et al.*, 1989c). Such an intimate adherence mechanism may also cause an issue for clinical therapy (Hill *et al.*, 1991, Law, 1994). In order to examine A/E lesion formation, electron microscopy (EM) was required before Knutton *et al.* (1989a) developed the fluorescent-actin staining (FAS) test. In this test, Fluorescein isothiocyanate (FITC) or Rhodamine (TRITC)-labelled phalloidin is used to specifically bind polymerized filamentous actin (F-actin) accumulated at the sites of bacterial attachment (Fig.1.2). This test has proved a very valuable tool for the study of bacterial factors involved in A/E lesion formation.

Both EPEC and EHEC are able to induce typical A/E lesions (Phillips and Frankel, 1997), and are therefore both AEEC, but they are not the only pathogens that can initiate A/E pathology as the mouse gastrointestinal pathogen, *Citrobacter rodentium* (CR) can also induce A/E lesions on infected host cells (Luperchio and Schauer, 2001).

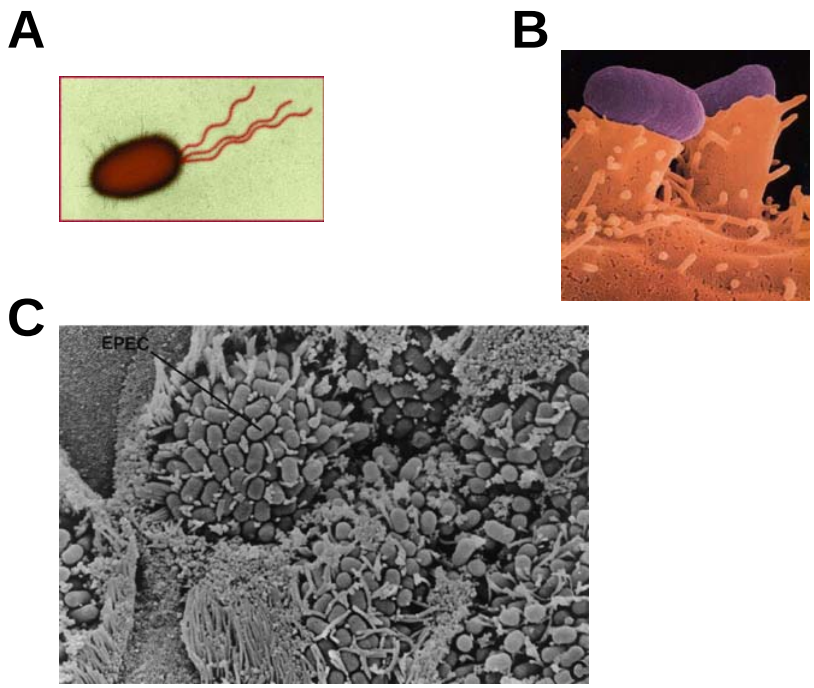


Fig. 1.1. A. Enterohaemorrhagic *Escherichia coli* (EHEC) (Image credit: Dennis Kunkel Microscopy, Inc.); B. EPEC (purple) induces the formation of pedestals on the host cell surface (orange) (Image reproduced from www.biotech.ubc.ca/faculty/finlay/); C. Gross localized perturbation of brush border architecture. Original magnification: ×8000. (Image reproduced from Frankel *et al.*, 1998)

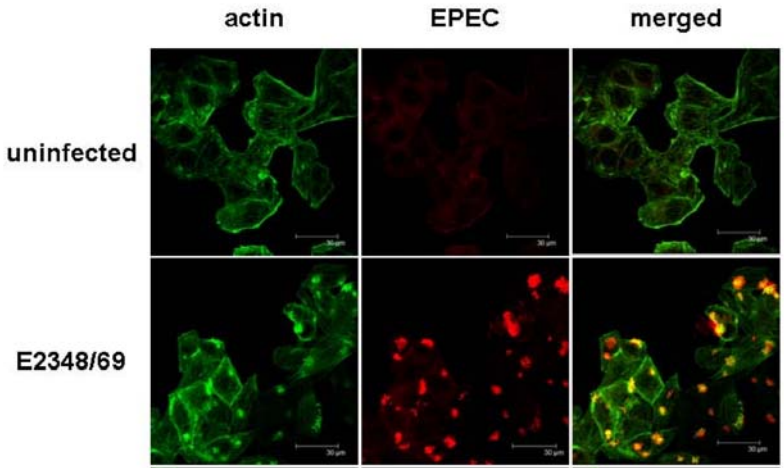


Fig. 1.2. Fluorescent-actin staining (FAS) test of EPEC infection.
EPEC strain E2348/69 was used to infect HEp-2 tissue culture cells. Actin (green) and EPEC bacteria (red) were detected after staining with Alexa 488 phalloidin and rabbit anti-EPEC polyclonal anti-sera, followed by anti-rabbit antibody labelled with Cy3 (reproduced from Hobson *et al*., 2008).

C. rodentium is the causative agent of transmissible murine colonic hyperplasia and is associated with mild diarrhoea in infected mice (Klapproth *et al.*, 2005). In the 1960s, *C. rodentium* was isolated after two major outbreaks of diarrhoea in laboratory mice in the United States (Brennan *et al.*, 1965) and Japan (Muto *et al.*, 1969) which resulted in high morbidity and mortality. At the time the isolates were designated as an atypical *Citrobacter freundii* strain (*C. freundii* ANL) or Murine pathogenic *Escherichia coli* (atypical *Escherichia coli* Ex30) respectively. Further studies on subsequent disease outbreaks in mouse colonies revealed a genomic relatedness among all atypical *C. freundii* and Murine pathogenic *Escherichia coli* strains isolated from rodents (Schauer *et al.*, 1995, Luperchio *et al.*, 2000). Therefore, they were reclassified as a separated species, *Citrobacter rodentium*. As with the human pathogens EPEC and EHEC, *C. rodentium* is able to form typical A/E lesions on infected intestinal tissue and serves as an important model pathogen for investigating the mechanisms controlling attaching and effacing pathology and it provides an ideal *in vivo* model for studying interaction between host and pathogen, including epithelial hyperproliferation and tumour promotion in the distal colon of the mouse (Luperchio and Schauer, 2001, Kelly *et al.*, 2006).

EPEC is a common cause of diarrhoea in developing countries nowadays, and particularly dangerous for children less than 1 year old (Hill *et al.*, 1991, Jarvis *et al.*, 1995, Frankel *et al.*, 1998). EPEC was first reported in the 1940s when an infantile diarrhoea outbreak in UK was investigated (Bray, 1945, Walker-Smith, 1996). However, during the following decades, there were doubts about the pathogenic potential of EPEC until 1978 when research involving human volunteers Levine *et al.*, (1978) demonstrated its

pathogenicity by inducing diarrhoea in the volunteers following ingestion of EPEC O127:H6 strain E2348/69. For many years, EPEC was defined by serotype only and a consensus was reached on the basic characteristics of EPEC in 1995 which included the capacity to form A/E lesions and the lack of Shiga toxin; as another closely related AEEC, EHEC, can produce Shiga toxin (McDaniel *et al.*, 1995).

1.4 Enterohaemorrhagic Escherichia coli (EHEC)

Enterohaemorrhagic *Escherichia coli* are associated with gastrointestinal infection in humans. EHEC strains are defined by a combination of factors including the ability to produce Shiga toxin(s), specific adherence factor(s), enterohaemolysin, in addition to the somatic antigens which the most prevalent EHEC strains express; including O111, O26 and O157. EHEC O157:H7 is the main EHEC serotype associated with outbreaks of gastrointestinal disease in North America, parts of Europe and Japan which are mainly developed countries. It is an important pathogen that can be life-threatening particularly in the young and the elderly. EHEC was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhoea in the USA and the outbreak was traced to contaminated hamburgers (CDC, 1982, Frankel *et al.*, 1998). Therefore, it was initially known as a "hamburger disease". Since then, EHEC have emerged as an important group of zoonotic pathogens (Table 1.1) that can cause acute gastroenteritis (an inflammation of the gastrointestinal tract resulting in acute diarrhoea) and haemorrhagic colitis (HC) (an inflammation of the large intestine with the loss of blood) (Noel and Boedeker, 1997, Croxen and Finlay, 2010, Dziva *et al.*, 2004, Rivas *et al.*, 1996, Roe, 2000), with the

potential to lead to haemolytic uremic syndrome (HUS) which is a severe complication characterized by hemolytic anemia (a decrease in the normal number of red blood cells because of the abnormal breakdown), acute renal failure (kidney damage) and thrombocytopenia (a low platelet count) (Corrigan and Boineau, 2001, Anagnou *et al.*, 1991, Bogdanovic *et al.*, 1988, Olgaard *et al.*, 1974, Riley *et al.*, 1983, Boyce *et al.*, 1995).

Table 1.1 Major outbreaks of EHEC infection since 1982

Time	Country	Details
1982	USA	Restaurant, contaminated hamburgers (CDC, 1982)
1982	USA	The food-distribution system contaminated spinach (CDC, 1982, Riley *et al.*, 1983)
Sept. 1984	Nebraska, USA	Nursing home; undercooked hamburger patties (Ryan *et al.*, 1986)
Sept. 1985	Canada	Nursing home; contaminated sandwiches and then a second wave associated with person-to-person transmission of infection (Carter *et al.*, 1987)
Apr. 1986	Canada	Dairy farm; a field trip to a dairy farm / raw milk (Dorn and Angrick, 1991)
Nov. 1986	Washington state, USA	Restaurant/nursing home; ground beef (Ostroff *et al.*, 1990)
1988	Germany	Multiple cases, no clear cource; sorbitol fermenting EHEC strain O157:H^- (Karch *et al.*, 1993)
Oct. 1988	Missouri, USA	day-care center; undercooked hamburger (Skala, 1994, Armstrong *et al.*, 1996)
Jan. 1990	Missouri, USA	>100 cases, the source of the outbreak has not been identified, may have been waterborne (Swerdlow *et al.*, 1992)
1990	Saitama, Japan	Water supply in a nursery (Akashi *et al.*, 1994)
1991	Massachusetts. USA	23 patient drank apple cider purchased at a roadside stand (Besser *et al.*, 1993)
Dec. 1991	Minnesota USA	Schools; meat supplied in the meals (Armstrong *et al.*, 1996)
May. 1992	Scotland UK	A semi-rural area of south-east Scotland; related to a childrens' paddling pool (Brewster *et al.*, 1994)
Dec. 1992 - Jan. 1993	The western USA	Restaurants; 700 people ill from *Escherichia coli* O157:H7 in hamburgers (Crump *et al.*, 2002)
Jul. 1993	California, USA	Home cooked hamburgers (CDC, 1994a & 1994b)
Mar. - Aug. 1993	Oregon and Washington, USA	4 chain Z restaurants: contaminated steak and salad (Jackson *et al.*, 2000)
1994	Leicestershire, UK	*Escherichia coli* O157:H7 isolated from nine animals on the farm was indistinguishable from the strain isolated from human samples (Shukla *et al.*, 1995)
Jul. 1994	New Jersey, USA	Meat (CDC, 1995a, CDC, 1995b)
1995	Wales UK	Farm; contact with cattle (Milne *et al.*, 1999)
Jul. 1995	Georgia and Tennessee, USA	Restaurant; hamburgers (CDC, 1996b)
Jul. 1995	northern Illinois, USA	12 children swimming at a bathing beach in Rock Cut State Park

		(CDC, 1996a)
Jul. 1996	Sakai City, Osaka, Japan	contaminated bean sprouts, >6000 cases occurred, after the outbreak, more than 1000 secondary infections occurred in the families of the patients (Yoshioka *et al.*, 1999)
Oct. 1996	North America	Unpasteurized apple cider or juice (McCarthy, 1996)
Oct. 1996	Morioka, Japan	198 students and seven school employees were infected with the O157 strain of *Escherichia coli*. Radish sprouts were briefly suspected in this outbreak (Watanabe *et al.*, 1999, Michino *et al.*, 1999)
Nov. 1996	Lanarkshire Scotland UK	500 people infected and at least 20 deaths. The outbreak was traced to a single butcher's shop and bakery which operated a substantial wholesale and retail trade in cooked and raw meat products (Dundas *et al.*, 1999, Attenborough and Matthews, 2000)
Jun. - Jul. 1997	Michigan and Virginia, USA	Associated with eating alfalfa sprouts grown from the same lot of seed (CDC, 1997)
Aug. 1997	USA	The outbreak was traced to the contaminated beef processed in a plant in Nebraska and distributed nationally (Bender *et al.*, 1997)
Jun. 1998	Nevada and California, USA	non-motile strain of *Escherichia coli* O157. Cases were linked to an alfalfa/clover sprout mixture from the same producer (Van Beneden *et al.*, 1999)
Jun. 1998	USA	50 people infected and cases linked to tainted cheese curds made at a Stanley dairy (CDC, 2000a & 2000b)
Aug. 1999	Washington County, New York, USA	consumption of beverages purchased from vendors supplied with water drawn from an unchlorinated fairground's well was associated with the outbreak (CDC, 1999)
May 2000	Scotland UK	Water supply as possible source (Artz and Killham, 2002)
Jul. - Sept. 2000	Wisconsin USA	Contaminated beef at Sizzler restaurant (Yoon *et al.*, 2009)
Dec. 2000	Minnesota USA	Contaminated ground beef purchased from SuperValu/Cub food stores (Proctor *et al.*, 2002)
2000	Pennsylvania, USA	Visiting a dairy farm (Crump *et al.*, 2002)
2000	Ohio, USA	The Medina County fair; the water system from which food vendors were supplied was the source of the *Escherichia coli* outbreak (Crump *et al.*, 2003)
Aug. 2001	British Columbia, Canada	Raw goat's milk from a co-operative farm (McIntyre *et al.*, 2002)
2001	Ohio, USA	An environmental and site investigation revealed *Escherichia coli* contamination on doorways, rails, bleachers, and sawdust. Investigators concluded that the Lorain County Fair was the source of the outbreak (Crump *et al.*, 2003)
Nov. 2001	northwestern England UK	Contaminated meat (Rajpura *et al.*, 2003)
Jul. 2002	Washington UK	Romaine lettuce made by 'Spokane Produce' (Lee *et al.*, 2004)
Aug. 2002	Lane County, Oregon USA	Originated from exposure to animals in 'the sheep and goat barn' (Services, 2003)
Aug. 2003	Dublin Ireland	Restaurants were identified as the possible source of the outbreak (Carroll *et al.*, 2005)
Sept.-Oct. 2003	California USA	Contaminated spinach (Jay *et al.*, 2007)
2003	Texas USA	25 people ill after attending the Fort Bend County Fair (Durso *et al.*, 2005)
2004	North Carolina USA	Associated with a goat and sheep petting zoo (Goode *et al.*, 2009)
Mar. 2005	Orlando, Tampa, USA	Associated with animal petting (CDC, 2005)
Sept. 2005	South Wales UK	A total of 157 cases were identified, mainly school children from contaminated meals (O'Brien, 2005)
Dec 2005	Washington & Oregon, USA	Raw milk (CDC, 2007)
May 2006	Scotland UK	Associated with a nursery (Pollock *et al.*, 2010)
Sept. 2006	USA	Raw spinach (Wendel *et al.*, 2009)

Nov. – Dec. 2006	USA	81 illness linked to Taco John's Restaurants
Sept. 2007	Scotland, UK	Contaminated cold meat (Webster *et al.*, 2007)
Jun, 2008	Ohio and Michigan, USA	Associated with ground beef sold at Kroger stores
Jun. 2009	USA	Outbreak linked to Nestle cookie dough
Aug. 2009	Surry England, UK	Visiting Godstone 'petting' farm (Wise, 2009)
Nov. 2009	New York, USA	Contaminated ground beef from a New York ground beef company (Moss, 2009)
Feb. 2010	England UK	Feltham Hill Nursery & Infant School, contamination source undefined (Teed, 2010)

EHEC infection is characterized by abdominal pain and contraction, followed by diarrhoea (Su and Brandt, 1995). As the disease progresses, the diarrhoea becomes watery or even bloody. Vomiting can occur, but rarely fever (Ryan *et al.*, 1986). The incubation period for the disease is normally 3 to 9 days, although shorter and longer periods can be observed. In general, this sickness lasts for about a week and resolves without any further long-term problems (Cohen and Giannella, 1992, Griffin *et al.*, 1988). Most infections have been traced back to contamination from ruminant faeces (polluted water, fruit and vegetables) (Hashimoto *et al.*, 1999). The other sources of infections include meat (ground beef), cross-contamination in food preparation, waterborne, person-to-person, animal-to-person and other foods of animal origin (Okrend *et al.*, 1990, Padhye and Doyle, 1991, Chapman *et al.*, 1993, Rangel *et al.*, 2005, Rivas *et al.*, 1996). A further factor, which is of great significance, is the size of the infectious dose (<700 organisms): this is incredibly small in comparison with those for most other food-borne pathogens (Tuttle *et al.*, 1999). *Escherichia coli* O157:H7 infection can be prevented simply by thoroughly cooking ground beef, avoiding unpasteurized milk and cross-contamination of foods, and washing hands carefully (VernozyRozand and RayGueniot, 1997). *Escherichia coli* O157:H7 infections can occur as isolated cases or as part of an outbreak (AlJader *et al.*, 1999, Tuttle *et al.*, 1999). Cases are seen more frequently

during the summer months which might due to high prevalence of EHEC contaminations in farm products and/or environmental survival of the bacteria (Chapman *et al.*, 1997, Heuvelink *et al.*, 1998, Van Donkersgoed *et al.*, 1999, Chapman *et al.*, 2000).

The natural reservoirs of EHEC are ruminants, such as cattle and sheep, in which EHEC do not seem to cause disease (Orskov *et al.*, 1987, Elder *et al.*, 2000). A 2003 study on the prevalence of *Escherichia coli* O157:H7 in livestock at 29 county and 3 large state agricultural fairs in the United States found that *Escherichia coli* O157:H7 could be isolated from 13.8% of beef cattle, 5.9% of dairy cattle, 3.6% of pigs, 5.2% of sheep, and 2.8% of goats (Keen, 2003). Over seven percent of pest fly pools also tested positive for *Escherichia coli* O157:H7. Although cattle are known to be a major reservoir for this pathogen, the ecology of the organism in animals is poorly understood. Four strains of O157:H7 were surveyed in experimentally infected calves by Naylor *et al.*. Most bacteria were found in faeces, and post-mortem examination revealed that the recto-anal tissues were colonised by *Escherichia coli* O157:H7 (Naylor *et al.*, 2003). These tissues also express a high density of lymphoid follicles, which may govern tropism, possibly via intimin - the bacterial adhesion protein (Naylor *et al.*, 2003). The accessibility and identification of this site of colonisation may facilitate simple intervention strategies.

1.4.1 Shiga toxin

A defining characteristic of EHEC is the production of Shiga toxin (Stx), the genes for which are introduced into strains on certain lambdoid

bacteriophages. While there are many factors that contribute to the pathogenesis of EHEC infection, Stx is a key virulence factor causing the most severe symptoms (O'Brien *et al.*, 1983, Karmali *et al.*, 1983). These phage-encoded toxins are comprised of a single A subunit (~32 KDa) associated with a pentamer of B subunits (~7.7 KDa) (Tesh and O'Brien, 1991). There are two groups of the Shiga toxin family which are called Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Stx1 is basically identical to Shiga toxin produced by *Shigella dysenteriae* type 1 (O'Brien *et al.*, 1982, O'Brien and LaVeck, 1983) which is also a cause of HUS. Stx2 has multiple subtypes (Stx2c, Stx2e, Stx2v etc.) which have different binding affinities to host cell molecules such as Globotriaosylceramide (Gb3) or Globotetraosylceramide (Gb4) (Lingwood *et al.*, 1987, Boyd and Lingwood, 1989, DeGrandis *et al.*, 1989, Lingwood, 1993).

It has been shown that purified Stx can cause fluid accumulation and histological damage in rabbit ileal loops (Keenan *et al.*, 1986). Considering the Gb3 receptor binding of Stx and that the Gb3 receptor is present at higher levels on villus cells rather than on crypt cells in rabbits, a possible diarrhoeagenic mechanism in this host is the selective killing of absorptive villus tip intestinal epithelial cells by Stx; however the distribution of the Gb3 receptor is different in human cells, and so the basis to EHEC induction of diarrhoea in humans is unclear and maybe more likely to be associated with host cell responses to other virulence factors (Schuller *et al.*, 2004, te Loo *et al.*, 2000). Stx is also referred to as Verocytotoxin (VT) or Shiga-like toxin (SLT) (Richardson *et al.*, 1988, O'Brien *et al.*, 1984). As a consequence, EHEC are considered a subset of the Verocytoxin producing *E.coli* (VTEC) or Shiga toxin producing *Escherichia coli* (STEC).

1.4.2 Type 3 secretion system (T3SS)

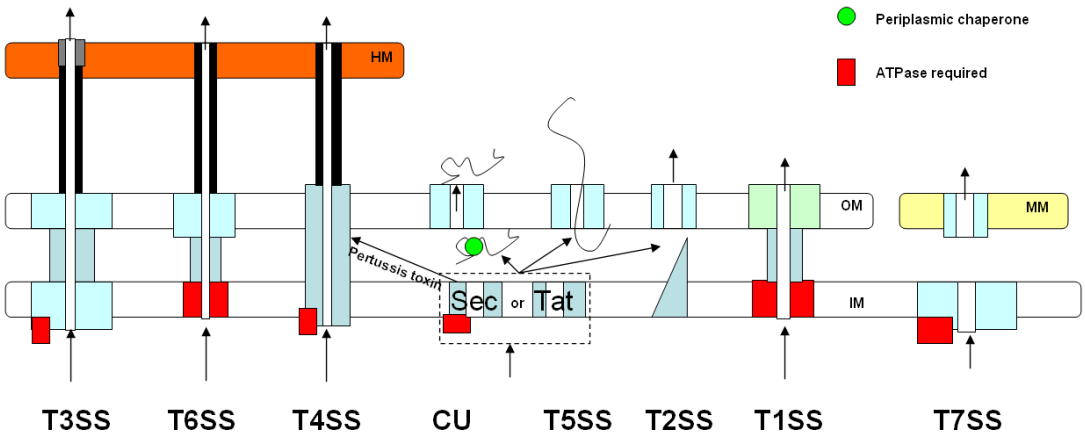


Fig. 1.3. Schematic diagram of bacterial secretion pathways.
It is only the basics of each secretion system that are presented here. HM: Host membrane; OM: Outer membrane; IM: Inner membrane; MM: Mycomembrane, ATPase is shown in red and periplasmic chaperone is shown in green.

At least seven different bacterial secretion systems have been reported and have been numbered accordingly; type 1, 2, 3, 4, 5, 6, and 7 secretion systems (Fig.1.3.). The type 1 secretion system (T1SS) is able to allow polypeptides of up to 800 kDa across the cell envelope which depends on an ATP-binding cassette (ABC) transporter in the inner membrane, a membrane fusion protein (MFP) and outer membrane protein (Omp) (Holland *et al.*, 2005). Type 2 secretion systems (T2SS) utilize a two-step process in which proteins are delivered to the periplasm first via the general secretory system (Sec) or potentially the Twin-arginine translocation (Tat) pathway and then transported to the exterior by an outer membrane secretin (Cianciotto, 2005). The type 4 secretion/pilus system (T4SS/T4PS) is evolutionally-related to T2S which utilize a simultaneous one step Sec-independent translocation of substrates across both membranes which can translocate protein, DNA or

protein-DNA complexs (Christie *et al.*, 2005). However, it was suggested that the translocation of Pertussis toxin subunits requires a Sec-dependent T4SS (Gauthier *et al.*, 2003b). The type 5 secretion system (T5SS) is an autotransporter system in which the protein, once in the periplasm, forms a pore-like complex to present part of its own polypeptide sequence externall, this can remain attached or be cleaved for export (Henderson *et al.*, 2004). The type 6 secretion system (T6SS) was discovered more recently and is not as well characterized yet (Filloux *et al.*, 2008). It can translocate virulence factors into eukaryotic cells in an ATPase (ClpV) dependent manner (Bingle *et al.*, 2008). Type 7 secretion systems have been found in Gram-positive and Acid fast bacteria for the secretion of the prototypic ESX proteins including the 6 kDa early secreted antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) (Simeone *et al.*, 2009). In addition to the above, the chaperone-usher pathway is employed to assemble various pili (Kline *et al.*, Nuccio and Baumler, 2007). The substrate requires the *sec* system for crossing the inner membrane and then the formation of a chaperone-substrate complex to prevent auto-aggregation in the periplams and to deliver the protein to the 'gateway' usher in the outer membrane for export (Thanassi *et al.*, 1998).

T3SS is a Sec-independent delivery channel for ATP-energized effector protein delivery into host cells, although the same system initially exports the translocation needle/filament and pore forming proteins to enable this export. In this respect, this export is equivalent to that used to drive flagellal export (Thomas and Finlay, 2003, Aldridge and Hughes, 2001), and in fact flagella in certain species have been associated with exporting effector proteins into host cells, e.g. from *Campylobacter* spp. (Young *et al.*, 1999,

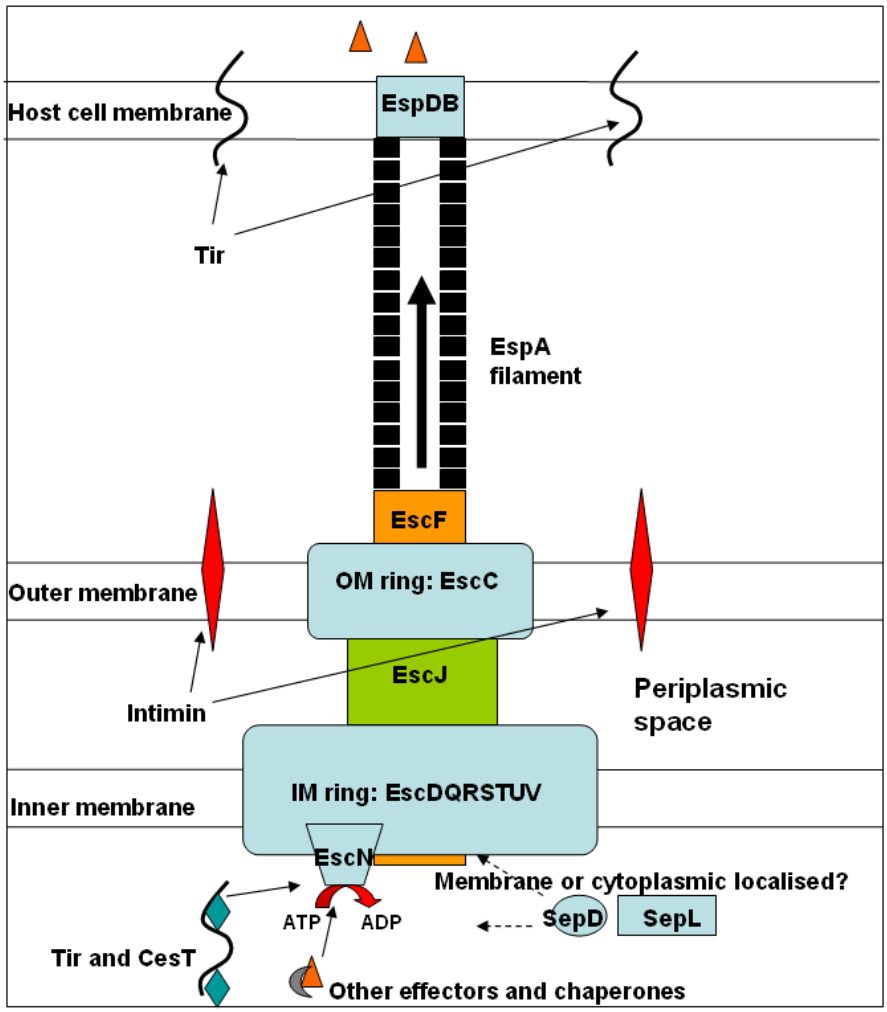


Fig. 1.4. Schematic diagram of the type three secretion system [Based on (Gauthier and Finlay, 2003, Gauthier *et al.*, 2003a, Yip *et al.*, 2005b, Crepin *et al.*, 2005a, Nadler *et al.*, 2006, Ogino *et al.*, 2006, Sekiya *et al.*, 2001, Daniell *et al.*, 2001, Wilson *et al.*, 2001, Andrade *et al.*, 2007, O'Connell *et al.*, 2004, Tree *et al.*, 2009)].
The basal body of the T3SS is composed of the outer membrane ring EscC, the inner membrane proteins EscD, Q, R, S, T, U, V, and the EscJ lipoprotein, which connects the inner and outer membrane ring structures. The needle structure, EscF, provides a base for EspA polymerization to form the filament. EspB and EspD form the translocation pore in the host cell plasma membrane, connecting the bacteria with the eukaryotic cell via EspA filaments. The ATPase EscN provides the energy to the system by hydrolyzing ATP molecules into ADP. The location of SepD and SepL remains unclear (Kresse *et al.*, 2000, O'Connell *et al.*, 2004).

Konkel *et al.*, 2004). The T3SS is composed of a basal apparatus spanning across the two bacterial double membranes and an extension structure to reach the host cell (Yip and Strynadka, 2006, Galan and Wolf-Watz, 2006,

Zarivach *et al.*, 2007, Zarivach *et al.*, 2008). In brief, T1, 3, 4 and 6 secretion systems can translocate substrates out of the bacterial cell in a one-step process; and the other systems need an intermediate step via the periplasm.

T3SS was first recognized in 1993 (Salmond and Reeves, 1993) after intensive work has been carried simultaneously on the Yop/Ysc secretion in an animal pathogen, *Yersina enterocolitica,* by two independent research groups led by Guy R. Cornelis and Hans Wolf-Watz respectively (Michiels *et al.*, 1990, Michiels and Cornelis, 1991, Forsberg *et al.*, 1991, Hakansson *et al.*, 1993, Cornelis and Wolf-Watz, 1997). Very soon after that, similar secretion machinery was also found in plant pathogens, *Erwinia amylovora* and *Pseudomonas solanacearum* (Wei *et al.*, 1992, Genin *et al.*, 1992, Gough *et al.*, 1992). The T3SSs are molecular syringes (needles) that inject bacterial virulence factors or effector proteins directly into host cells. These injected bacterial molecules subvert cellular processes and contribute to the disease. As with other Gram-negative pathogens, such as *Yersinia* spp., *Pseudomonas* spp., *Shigella* spp. and *Salmonella* spp., EHEC has a type 3 secretion system (T3SS) (Fig. 1.4) (Table 1.2) (Urbanowski *et al.*, 2005, Torruellas *et al.*, 2005, Schroeder and Hilbi, 2008, Miki *et al.*, 2009).

EHEC T3SS consists of the structural proteins known as the basal apparatus (EscCDJRSTUV…) and also the translocon proteins (EspADB) which form a needle like filament to facilitate injection of effectors into the host cells (Ogino *et al.*, 2006). The basal apparatus consists of multiple proteins forming a pore through the bacterial double membrane which requires the Sec-pathway for assembly. The basal appratus recruits effector proteins to the export channel with the help of chaperone proteins (Gauthier and Finlay,

2003, Gauthier *et al.*, 2003a, Thomas *et al.*, 2005, Zarivach *et al.*, 2007). With the help of the apparatus ATPase protein - EscN, effectors are unfolded and energized for release through the T3SS 'tunnel' (Akeda and Galan, 2005, Evans *et al.*, 2006, Gauthier and Finlay, 2003, Gauthier *et al.*, 2003a, Zarivach *et al.*, 2007, Andrade *et al.*, 2007). After then it was proposed substrate export would be driven by the proton motive force (PMF) in the T3SS 'tunnel' (Galan, 2008, Minamino and Namba, 2008, Paul *et al.*, 2008). All the structural proteins are crucial for the T3SS and any damage of these proteins would result in a severe impairment of T3SS function (Gauthier *et al.*, 2003a, Nadler *et al.*, 2006, Ogino *et al.*, 2006). The ATPase is required for T3S and this has been shown in a number of studies (Gauthier and Finlay, 2003, Zarivach *et al.*, 2007, Andrade *et al.*, 2007). The PMF is known to drive ion, DNA and protein/solute transport in many different cases (Ohsumi and Anraku, 1983, Wong and Buckley, 1989, Strobel *et al.*, 1989, Van Leeuwen *et al.*, 1991, Bradbeer, 1993, Bose *et al.*, 2002, Maier *et al.*, 2004, Crosa *et al.*, 2009) and was suggested to be involved in flagellar assembly back in 1977 (Bar Tana *et al.*, 1977) and subsequent work proved this theory (Galperin *et al.*, 1982, Paul *et al.*, 2008). Recent work has suggested the PMF as important for T3S in *Yersina* but the mechanism remains to be elucidated (Wilharm *et al.*, 2004).

Exo-flagella are whip-like organelles emanating from the bacterial surface which are important for bacterial motility and pathogenesis (Karlinsey *et al.*, 1998, Karlinsey *et al.*, 2000a, Eichelberg and Galan, 2000, Giron *et al.*, 2002, Berg, 2003). It was demonstrated that the bacterial flagellar hook and filament were assembled in a manner analogous to that of the T3SS (Karlinsey *et al.*, 2000a, Desvaux *et al.*, 2006). Published work confirmed

that flagellar T3SS (F-T3SS) and non-flagellar T3SS (NF-T3SS) were all probably derived from the same ancestor (Nguyen *et al.*, 2000, Gophna *et al.*, 2003, Saier, 2004). There were at least nine conserved proteins shared between the F-T3S and NF-T3S systems (Desvaux *et al.*, 2006). However,

Table 1.2 Non-flagellar T3SSs found in various Gram-negative pathogens (Urbanowski *et al.*, 2005, Torruellas *et al.*, 2005, Kelly *et al.*, 2006, Schroeder and Hilbi, 2008, Miki *et al.*, 2009, Wang *et al.*, 2009, Cornelis, 2006)**.**

T3SS family	Taxon	Organism	Details
Ysc	γ -proteobacteria	*Yersina pestis*	Human, cattle, rodent pathogen
		Yersinia pseudotuberculosis	
		Y. enterocolitica	Human pathogen
		Pseudomonas aeruginosa	Animal, insect and human, Opportunistic pathogen
		Vibrio parahaemolyticus	Human pathogen
		Aeromonas salmonicida	Fish pathogen
		Photorhabdus luminescens	mutualistic with entomophagous nematodes
	β -proteobacteria	*Bordetella pertussis*	Human pathogen
	δ -proteobacteria	*Desulfovibrio vulgaris*	Sulphate reducing environmental bacteria
SPI-2	γ -proteobacteria	EPEC, EHEC	Human pathogens form AE lesion
		Citrobacter rodentium	Mouse pathogen form AE lesion
		Salmonella enterica	Human pathogen (SPI-2 responsible for Intracellular lifecycle)
		Yersina pestis	Rodent and human pathogen
		Yersinia pseudotuberculosis	
		Edwardsiella tarda	Human pathogen
	β -proteobacteria	*Chromobacterium violaceum*	Emerging human pathogen (evoking meloidosis)
SPI-1	γ -proteobacteria	*Salmonella enterica*	Human pathogen (SPI-2 responsible for host cell invasion)
		Shigella flexneri	Human pathogen
		Yersinia enterocolitica	Human pathogen
		Sodalis glossinidius	Tse-tse fly symbiont
	β -proteobacteria	*Burkholderia pseudomallei*	Human pathogen
		Chromobacterium violaceum	Emerging human pathogen (evoking meloidosis)
Hrp1	γ -proteobacteria	*Pseudomonas syringae,*	Plant pathogen
		Erwinia amylovora	Plant pathogen
		Pantoea agglomerans	Non-pathogen, environmental and human commensal
		Vibrio parahaemolyticus	Human pathogen
Hrp2	γ -proteobacteria	*Xanthomonas campestris*	Plant pathogen
	β -proteobacteria	*Ralstonia solanacearum*	Plant pathogen
		Burkholderia pseudomallei	Human pathogen
Chlamydiales	Chlamydiaceae	*Chlamydia trachomatis*	Obligate intracellular human pathogen
		Chlamydia pneumoniae	Obligate intracellular human pathogen
Rhizobium	α -proteobacteria	*Mesorhizobium loti*	Plant symbiont (Nitrogen fixation)
		Rhizobium sp	Plant symbiont (Nitrogen fixation)

Table 1.3 Main components of T3SSs

Organism	Filament	ATPase	Needle	Gatekeeper	Ruler	Switch	C-ring
Yersina spp. (Ysc)	LcrV	YscN	YscF	YopN-TyeA	YscP	YscU	YscQ
EHEC/EPEC	EspA	EscN	EscF	SepL	Orf16	EscU	EscQ
Salmonella enterica (SPI-1)	SipD	InvC	PrgI	InvE	InvJ	SpaS	SpaO
Shigella spp.	IpaD	Spa47	MxiH	MxiC	Spa32	Spa40	Spa33
Flagellum	FliC	FliI	FlgE	-	FliK	FlhB	FliN

the F-T3SS and NF-T3SS are not the exact same system but evolutionary related (Table 1.3) (Nguyen *et al.*, 2000, Gophna *et al.*, 2003, Saier, 2004). Structurally, both systems have a basal complex spanning between two bacterial membranes with a protein export channel in the middle; they also have a hollow filamentous organelle extended from the basal complex (Bar Tana *et al.*, 1977, Wei *et al.*, 2001, Giron *et al.*, 2002). Functionally, the flagellar filament is flexible and can rotate driven by the PMF which is critical for bacterial motility (Journet *et al.*, 2005, Bar Tana *et al.*, 1977); but the NF-T3SS normally has a needle like filament for effector translocation into host cells (Journet *et al.*, 2005). Generally, the non-flagellar T3SS is characterized by a number of attributes (i) contact-dependence (Mecsas and Strauss, 1996); (ii) an energy requirement for protein secretion and translocation into host cells; (iii) secretion-regulated expression of genes encoding proteins secreted downstream in the pathway; and (iv) dedicated cytoplasmic chaperones for some secreted proteins. It differs from other secretion pathways in Gram-negative bacteria by the absence of (i) conserved sequences involved in translocation except within some species (Miao, 2000), (ii) a cleaved signal sequence in secreted polypeptides, and (iii) a periplasmic secretion intermediate (Charkowski *et al.*, 1997). The non-flagellar and flagellar T3SSs share all but the first characteristic and the ability to translocate proteins into eukaryotic cells, although translocation may occur through flagella in certain bacteria. Another difference is that the group of YopN-TyeA family gating proteins that switch substrate secretion in the non-flagellar T3SS are not found but not in the flagellar system (Pallen *et al.*, 2005b). On the other hand, the flagella T3SS is closely regulated by sigma factors and chemotaxis (Paul *et al.*, 2010, Sarkar *et al.*,

2010, Dong and Schellhorn, 2009a, Aldridge *et al.*, 2006, Desvaux *et al.*, 2006, Dutton *et al.*, 2005, Tart *et al.*, 2005).

In contrast to the secretion process in other systems, T3S is triggered when a pathogen comes in close contact with host cells, and hence has been called 'contact-dependent secretion' (Ginocchio *et al.*, 1994, Watarai *et al.*, 1995, Menard *et al.*, 1994, Rosqvist *et al.*, 1994). Levels of type 3 protein secretion are also dependent on environmental cues (Kenny *et al.*, 1997a). Temperature, growth phase and salt conditions are factors known to induce synthesis of the secretion apparatus and effector proteins in various pathogens (Edwards and Schifferli, 1997, Chilcott and Hughes, 2000, Cambronne and Schneewind, 2002, Knodler *et al.*, 2002, Yahr and Wolfgang, 2006). Because of this medium-dependent secretion proteins are secreted only when the bacteria are cultured in a medium with the appropriate stimuli; absence of this information can make the secretion system difficult to study.

1.4.3 A pathogenicity Island (PAI) for A/E lesion formation

A three-stage model of pathogenesis was proposed by Donnenberg and Kaper for EHEC and EPEC (Donnenberg and Kaper, 1992). In this model: (1) with the help of bacterial surface adhesive factors, such as flagella or fimbriae, bacteria initially adhere to epithelial cells; (2) adherence of bacteria to epithelial cells induces different signal transduction pathways via injection of virulence factors that alter the intracellular environment in host cells; and (3) intimate bacterial adherence occurs to host cells with pedestal formation.

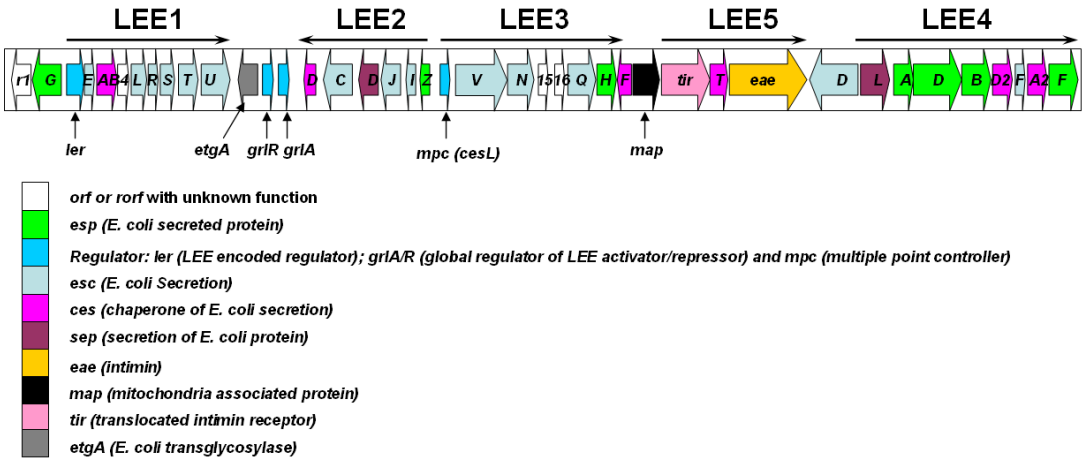


Fig. 1.5. Gene map of EHEC LEE
EHEC LEE has a size of about 35 Kbs and has 5 major operons with 41 genes. Those genes encode different components which contributed to E. coli type three secretion system including Basal apparatus (EscCDEIJNLQRSTUV), needle structure EscF, EspA filament and EspDB pore forming protein, Sep (secretion of *E. coli* protein) proteins, Ces (Chaperone of *E. coli* secretion) proteins, regulators, bacterial adhesion - intimin, traslocated effector (Map, Tir, , EspFGHZ) and other proteins with unknown function.

The capacity to form A/E lesions is conferred by a pathogenicity island known as the locus of enterocyte effacement (LEE) (Fig. 1.5.). As shown for EPEC O127, all the proteins needed for inducing A/E lesions can be encoded by this chromosomally-located PAI (McDaniel and Kaper, 1997). Previous research demonstrated that an *Escherichia coli* K12 strain can form A/E lesion on epithelial cells when transformed with the LEE from EPEC but, interestingly, not when transformed with the EHEC LEE (McDaniel and Kaper, 1997, Elliott *et al.*, 1999b). The size of the LEE in EPEC/EHEC O157 is about 35 kb (Elliott *et al.*, 1999b, Kaper *et al.*, 1997, Perna *et al.*, 1998, Frankel *et al.*, 1998). There are five major operons termed LEE1, LEE2, LEE3, LEE4 and LEE5 (including *tir*) in the LEE of EHEC, containing at least 41 open reading frames (ORFs).

These genes encode different products, ① Type 3 secretion apparatus: 'Esc' - *Escherichia coli* secretion and 'Sep' - secretion of *Escherichia coli* proteins;

② Type 3 ‘translocators’ including *Escherichia coli* secreted proteins (Esp)A,D,B; ③ effector proteins including the mitochondria-associated protein (Map), EspF/G/G2/H, and the translocated intimin receptor (Tir); ④ the outer membrane adhesin, Intimin; ⑤ transcriptional regulators: the LEE-encoded regulator (Ler); global regulator of LEE-activator/repressor (GrlA/R) and multiple point controller (Mpc); ⑥ chaperone proteins: CesT, CesAB, CesA2, CesD,CesD2 and CesF; ⑦ proteins of unknown function. The G+C content of the LEE (38.3%) is significantly lower than that of the *Escherichia coli* chromosome (50.8%) as a whole, suggesting that the LEE arose by horizontal gene transfer of this pathogenicity island from another species (Deng *et al.*, 2001, McDaniel and Kaper, 1997, Karaolis *et al.*, 1997).

1.4.3.1 Intimin (bacterial surface adhesion)

As mentioned above, the LEE in attaching and effacing pathogens encodes an important outer membrane adhesin, intimin, which binds to the bacterial protein Tir, as well as to host encoded proteins such as nucleolin on host cell membranes (Sinclair and O'Brien, 2002, Sinclair and O'Brien, 2004, Sinclair *et al.*, 2006, Kenny *et al.*, 1997b). These intimin-related interactions were demonstrated to play a pivotal role in inducing typical A/E lesions (Rosenshine *et al.*, 1996b, Frankel *et al.*, 1996, Kenny and Finlay, 1997, Kenny *et al.*, 1997b, Hicks *et al.*, 1998, Sinclair and O'Brien, 2002). Surface interactions are critical for the intimate attachment phenotype and trigger consequent cytoskeleton rearrangement in epithelial cells. So far, six intimin derivatives (α, β, γ, δ, ε and ξ) have been shown to be expressed by A/E pathogens (Jores *et al.*, 2003). Although the mechanism of intimin secretion was unknown, it was reported that intimin export required multiple

periplasmic chaperones and possibly a type 5 secretion system (Bodelon *et al.*, 2009).

1.4.3.2 T3 basal structure

As mentioned above the T3 needle complex (NC) shares similarities with the bacterial flagella system. The T3 NC starts at the cytoplasm of the bacterium, crosses the two membranes and protrudes out of the cell. This sophisticated injectisome can be considered in two parts; the basal body and polymerized EspA needle extension. The T3 basal apparatus is embedded in the bacterial membrane and forms a multi-ring structure spanning from the bacterial inner membrane to the outer membrane. This basal complex is composed of more than 20 structural proteins which are encoded by genes located within the LEE. The assembly of T3 NC involves three steps: First, the insertion of inner membrane and outer membrane ring in a Sec-dependent manner (Gauthier *et al.*, 2003a); and then export of the needle structural protein, EscF, that is integrated into the outer and inner rings as a base for the filament structure (Sekiya *et al.*, 2001, Daniell *et al.*, 2001, Wilson *et al.*, 2001). EspA is secreted via the T3SS and is added to the top of the EscF needle; polymerized EspA forms the main translocation filament (Daniell *et al.*, 2001).

There are many T3 basal proteins found in the EHEC inner membrane (Gauthier *et al.*, 2003a). These proteins include EscD (also known as Pas - protein associated with secretion), EscQ, EscR, EscS, EscT, EscU, EscV and the membrane associated ATPase – EscN (Kresse *et al.*, 1998, Ogino *et al.*, 2006, Pallen *et al.*, 2005a, Zarivach *et al.*, 2008, Gauthier and Finlay, 2003, Gauthier *et al.*, 2003a, Andrade *et al.*, 2007). EscD exhibits homology to the

T3 apparatus protein YscD of *Yersinia enterocolitica* and PscD of *Pseudomonas aeruginosa*. YscD is an inner membrane component in the *Yersinia* T3SS and mediates the delivery of virulence factors through the bacterial double membranes (Kresse *et al.*, 1998, Pallen *et al.*, 2005a). As a homologue protein in EHEC and EPEC, EscD was shown to be an inner membrane component and to be required for EHEC T3 export regulation (Kresse *et al.*, 1998). In this same study, direct interactions were detected between secreted proteins (Tir / EspD, B) and cytoplasmic but not membrane-localized EscD which hints at a potential mechanism of T3SS regulation (Kresse *et al.*, 1998). Further study of T3 basal apparatus assembly revealed that EscD could interact with EscC (outer membrane ring) and EscF (needle construct). EscC was proposed as an outer membrane ring protein and its localization has been proven in several reports (Gauthier *et al.*, 2003a, Ogino *et al.*, 2006). Using CsCl fraction analysis, EscC was found mainly in both the inner and outer membrane fractions (Ogino *et al.*, 2006) and could bind to EscD and EscF. Another predicted component of the basal apparatus, EscJ, is presumed to be exported into the periplasm in a Sec-dependent manner and to form a bridge spanning the two membrane rings, although this mainly localized to the outer membrane fraction (Crepin *et al.*, 2005a). EscJ was also known to bind to the needle protein, EscF, *in vitro* (Ogino *et al.*, 2006), providing a link between the translocon proteins and the basal apparatus (Sekiya *et al.*, 2001, Ogino *et al.*, 2006).

1.4.3.3 EspA filament

EspA filaments act as a bridge between the bacteria and host cells and effector proteins are translocated into host cells via this channel. EspA is a T3 translocon protein encoded by LEE which is polymerized to form a

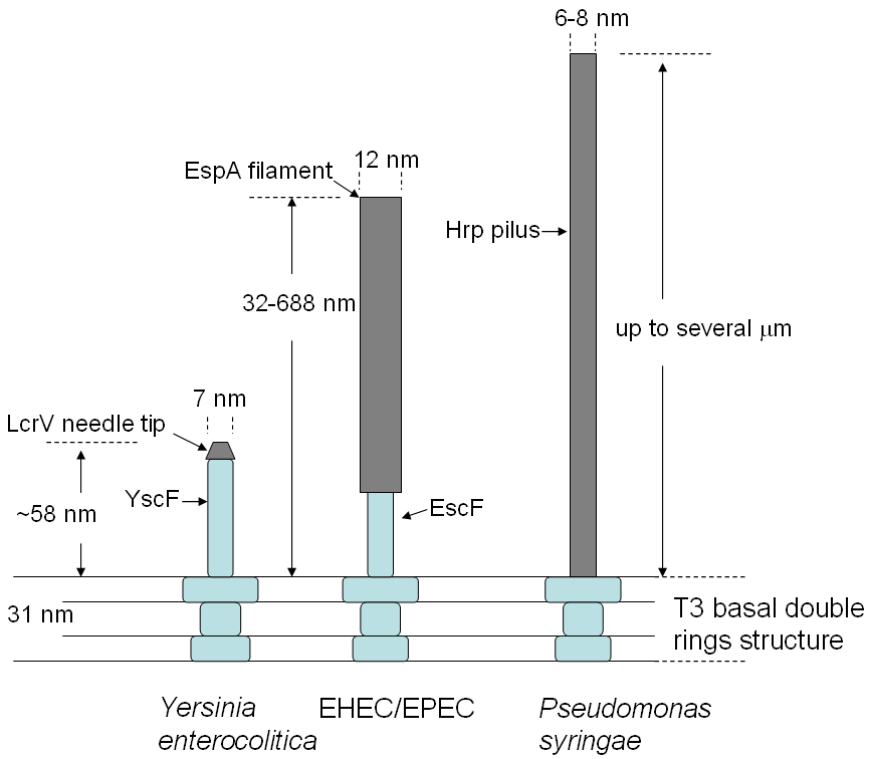


Fig. 1.6. Schematic diagram of EspA filament.
Comparing with the Ysc T3SS needle (Mueller *et al.*, 2005), the length of EHEC/EPEC EspA filament is highly variable and longer. EHEC/EPEC T3SS needles length was found in a range of 32-688nm (Sekiya *et al.*, 2001). For plant pathogen (*P. syringae*), the length of its Hrp pilus can be up to a few μm (Cornelis, 2006).

hollow conduit on the bacterial outer surface. Contact between the EspA filament and the host cell triggers completion of assembly of the filament tip during which EspD and EspB are secreted and inserted into the host cell membrane (Warawa *et al.*, 1999, Ide *et al.*, 2001).

EspA is a 25KDa peptide polymerized to form a helical tube and a whole EspA filament extends from the T3 basal apparatus by attaching to the EscF needle complex with EspD and EspB pore-forming proteins on the tip of the filament. The first report of a 3D structure of EspA filaments in EPEC was presented by Daniell *et al.* (2003). The structure comprises a helical tube which has a diameter of 120 Å with a central channel of 25 Å (diameter)

through which effector proteins may be transported (Knutton *et al.*, 1998, Cleary *et al.*, 2004). The subunit arrangement corresponds to a one-start helix with 28 subunits present in five turns of the helix and an axial rise of 4.6 Å per subunit (Daniell *et al.*, 2003). Unlike NF-T3SS needle extensions in other animal pathogens, the length of EspA filament is variable and much longer (Fig.1.6.) which implies its unique application in the gastrointestinal tract, for example for penetrating the mucus barrier and thick glycocalyx (Daniell *et al.*, 2001). Despite the similarity between EspA proteins in EHEC/EPEC, they show antigenic polymorphism as EspA filaments from different EPEC and EHEC strains show little immunological cross-reactivity (Neves *et al.*, 2003b, Crepin *et al.*, 2005b). Such polymorphisms among functional EspA filaments of EPEC and EHEC are likely to have important implications for the development of EspA-based vaccines.

EspD/B both have homology to YopD/B (*Yersinia)* and PopD/B (*Pseudomonas*) proteins (Wachter *et al.*, 1999, Broms *et al.*, 2003) which are thought to form a pore complex in the host membrane and induce contact-dependent haemolysis of red blood cells (RBCs) (Hakansson *et al.*, 1993, Rosqvist *et al.*, 1995, Hartland *et al.*, 1996, Neyt and Cornelis, 1999, Broms *et al.*, 2003). Previous studies have shown that EPEC induces contact-independent haemolysis of RBCs, and Esp translocon proteins are involved in this activity (Warawa *et al.*, 1999, Shaw *et al.*, 2001, Shaw *et al.*, 2002). Moreover, with EspA filaments opening a channel between the bacterium and host cell, a series of subsequent responses of the T3SS might be initiated in the pathogenic bacterium via sensing signals from the host cell (Abe *et al.*, 2002, Ide *et al.*, 2003, Roe *et al.*, 2003a).

1.4.3.4 T3 effector proteins

Various substrates are translocated into the host cell via T3SS at different stages of colonisation/infection. These 'effectors' contribute to different aspects of the host-pathogen interaction. In general, there are three main themes of activities related to T3SS translocated effectors. 1. Colonisation and invasion: EHEC/EPEC translocate their own bacterial adhesion receptor (Tir) into the host cells that allows intimate contact between bacteria and epithelia cells (Kenny *et al.*, 1997b). Other effectors, such as Map and EspG/G2 from EHEC/EPEC, EseG from *Edwardsiella tarda*, are involved in disruption of the host microtubule network which can be critical for pedestal formation (Dean and Kenny, 2004, Tomson *et al.*, 2005, Xie *et al.*). 2. Cytotoxicity: Some virulence factors can also cause tissue damage/dysfunction which might lead to cell death. For example, ExoU from *P. aeruginosa* was shown to be responsible for destruction of intracellular membranes (Veesenmeyer *et al.*, 2010). Translocated EspF from EHEC/EPEC can disrupt epithelial tight junction which contribute to diarrhoea symptoms (Crane *et al.*, 2001). 3. Immune-response: For example, NF-κB is a key factor activating the expression of inflammatory genes and T3 effectors can modulate host immune-responses by inhibiting its activation. SopE from *Salmonella*, OspG from *Shigella* and NleH from EPEC/EHEC are all involved in these activities (Friebel *et al.*, 2001, Kim *et al.*, 2005, Garcia-Angulo *et al.*, 2008).

1.4.3.4.1 LEE-encoded effector proteins

The EspA filament provides a channel for delivering T3S related effector proteins into eukaryotic cells. Many virulence factors are found encoded by genes of the LEE pathogenicity island which include Tir (EspE), Map, EspF,

G, H and SepZ (EspZ). These factors are injected into host cells and considered then to interfere with host cell regulatory pathways and functions. *orf19* (*map*) codes for the mitochondrial-associated protein (Map), which is transferred by the T3SS into host cells. Map plays at least two roles when it is injected into host cells. One is mediating Cdc42-dependent filopodia formation and another is targeting mitochondria to induce dysfunction (Czerucka *et al.*, 2001, Kenny *et al.*, 2002, Dean and Kenny, 2004, Dean *et al.*, 2006). Gene *espG* (*rorf2*) is highly conserved and can be found in the LEE of all A/E pathogens. EspG is normally expressed in very small amounts. It is speculated that EspG may play an accessory role in virulence. The function of EspG is not fully understood but it has been shown that EspG and its homologue EspG2 (*orf3*) were responsible for epithelial paracellular permeability and microtubule disruption (Elliott *et al.*, 2001, Tomson *et al.*, 2005, Matsuzawa *et al.*, 2005). EspH is encoded by *orf18* of the LEE and the translocation of EspH into host cells is T3SS-dependent. It is reported that EspH appeared to be localized to the cell membrane in host cells. Overexpression of EspH repressed the formation of filopodia and enhanced the formation of actin pedestals (Tu *et al.*, 2003). This fact indicates that EspH is a modulator of the host actin cytoskeleton structure. Effector protein SepZ, also known as EspZ, is hypervariable among A/E pathogens, with sequences sharing between 60 to 81% amino acid identity with SepZ of EPEC. (Kanack *et al.*, 2005) However, its location and function is still unknown in the host cell.

As one of the most thoroughly investigated effector proteins, Tir was the first EPEC effector molecule to be identified (Kenny *et al.*, 1997b) which was also known as Hp90 or EspE protein in previous reports (Rosenshine *et*

al., 1992, Deibel *et al.*, 1998). Tir is encoded by a gene in LEE5 which contains the bacterial adhesin (Intimin) gene – *eaeA*. It was a key finding that EPEC adherence is related to the tyrosine phosphorylation of a protein of ~ 90 KDa (Hp90) with subsequent studies revealing that Hp90-Intimin interaction was essential for pedestal formation (Rosenshine *et al.*, 1996a, Rosenshine *et al.*, 1996b, Kenny and Finlay, 1997). Although comparison of the EHEC O157 and EPEC O127 LEE regions shows a high degree of identity between the genes encoding the type 3 secretion apparatus and protein chaperons, those genes encoding the secreted and effector proteins are more variable (Perna *et al.*, 1998). Tir is the most divergent LEE-encoded molecule, with <60% identity between EHEC O157:H7 and EPEC homologues and only 40% identity within the C-terminal domain (Paton *et al.*, 1998, Kenny, 1999). The interaction of EPEC Tir with the outer membrane adhesin, intimin, triggers actin aggregation beneath the attached bacteria and Tir tyrosine/474 phosphorylation is essential for this event (Kenny, 1999). However, the EHEC O157:H7 Tir molecule is not tyrosine phosphorylated. Tir phosphorylation differences between enterohaemorrhagic and enteropathogenic *Escherichia coli* implied a different Tir-based mechanism for pedestal formation (DeVinney *et al.*, 2001).

Following being secreted or translocated into epithelial cells by T3 machinery, Tir_{EPEC} is integrated into the epithelial cell plasma membrane (Hartland *et al.*, 1999) exposing an extracellular central domain that functions as an intimin receptor. Interaction with intimin initiates clustering of Tir_{EPEC}, activating signalling pathways by recruiting the Nck/N-WASP/Arp2/3 complex followed by actin polymerization (Calier, 2000,

Campellone *et al.*, 2002, Campellone and Leong, 2003, Schuller *et al.*, 2007). As Tir_{EHEC} is not tyrosine phosphorylated, it was demonstrated to lack the Tir_{EHEC}/Nck interaction during pedestal formation. Later it was revealed that signalling activation in the host cell during EHEC infection requires recruitment of an EHEC T3 translocated factor ─ EspFu (TccP), an Nck-like protein (Campellone *et al.*, 2004, Garmendia *et al.*, 2004, Allen-Vercoe *et al.*, 2006) but binding to Tir via IRSp53/ Insulin receptor tyrosine kinase substrate (IRTKS) (Vingadassalom *et al.*, 2009, Weiss *et al.*, 2009). However, in certain EHEC and EPEC strains, a T3 effector TccP2 was found in complex with Tir to induce actin polymerization in addition of Nck signalling cascade (Whale *et al.*, 2006).

1.4.3.4.2 Non-LEE encoded effector proteins

Although the LEE encoded most of the proteins required for A/E lesion formation, many factors encoded by genes outside of the LEE contribute to pathogenesis (Morabito *et al.*, 2003). In 2003, a T3 secreted protein, Cif, was identified outside of the LEE in EPEC/EHEC using transposon mutagenesis and reporter gene studies (Marches *et al.*, 2003, Charpentier and Oswald, 2004). This molecule blocks cell cycle G2/M transition and induces the formation of stress fibres through the recruitment of focal adhesions (Marches *et al.*, 2003). Later, another Non-LEE-Encoded (Nle) type 3 translocated virulence factor, NleA (EspI), was found to be present in the LEE-containing *Escherichia coli* pathogens and *C. rodentium* using a proteomic analysis (Gruenheid *et al.*, 2004, Mundy *et al.*, 2004b) It was found absent from non-pathogenic strains of *Escherichia coli* and non-LEE-containing pathogens (Mundy *et al.*, 2004b). It was found to target to the Golgi in the host cell (Gruenheid *et al.*, 2004) and to inhibit cellular protein

secretion by disrupting mammalian COPII function (Kim *et al.*, 2007). Soon after that, multiple non-LEE encoded effectors (NleB-G) were identified in *C. rodentium* using a proteomic approach due to a T3SS-dependent hypersecretion phenotype exhibited by *sepL* or *sepD* mutants (Deng *et al.*, 2004). As most of Non-LEE-Encoded factors were discovered recently, only a few of them have been investigated thoroughly. Subsequent research revealed that NleB is found not be secreted into the bacterial supernatant while it was translocated into host cells and essential for colonisation (Kelly *et al.*, 2006, Roe *et al.*, 2007). Previously those effectors were identified using experimental approach, such as transposon mutagenesis, proteomic analysis and various reporters (Deibel *et al.*, 1998, Marches *et al.*, 2003, Deng *et al.*, 2004). In 2006, a collaboration between Toru Tobe and Mark J. Pallen's groups employing both bioinformatics and experimental methods (Tobe *et al.*, 2006) identified a large number of T3S effectors. There were 62 effector genes found using homology-based searches and 39 of them were proven to be exported using reporter genes and proteomic analysis (Tobe *et al.*, 2006). This study also discovered that most of those effector genes were found located in lambdoid prophages although they were scattered throughout the EHEC chromosome in 25 exchangeable effector loci (Tobe *et al.*, 2006).

1.4.3.5 T3 chaperone proteins

Many T3 secreted proteins depend on specific chaperones for stabilization and/or secretion. Chaperones identified for effector proteins of A/E pathogens so far include: CesT (Tir, Map, NleA…) (Abe *et al.*, 1999, Elliott *et al.*, 1999a, Creasey *et al.*, 2003a, Thomas *et al.*, 2005) and CesF (EspF) (Elliott *et al.*, 2002); and for the translocator proteins— CesD (EspB and

Table 1.4 Representative members of different classes of chaperones (Modified from Parsot *et al.*, 2003)

Class	Orgnism	Chaperone	Size	pI
IA	EPEC/EHEC	CesT	156	
	Yersinia spp.	SycE	130	4.5
		SycH	143	4.8
		SycT	130	4.4
		SycN	123	5.1
	Shigella spp.	IpgE	120	4.0
	Salmonella spp.	SicP	116	3.9
		SigE	113	3.9
	Pseudomonas spp.	SpcU	137	4.4
		Orf1	116	5.0
IB	EPEC/EHEC	CesF	127	4.2
	Shigella spp.	Spa15	133	4.2
	Salmonella spp.	InvB	135	4.4
	Yersinia spp.	YscB	137	9.3
		YsaK	135	4.3
	Sodalis spp.	InvB	135	3.7
II	EPEC/EHEC	CesD	151	7.4
		CesD2	135	5.3
	Yersinia spp.	SycB	168	4.5
		SycD	169	4.5
	Shigella spp.	IpgC	155	4.4
	Salmonella spp.	SicA	165	4.6
		SscA	157	8.0
	Pseudomonas spp.	PcrH	168	4.4
III	*Salmonella* spp.	FlgN	140	5.3
		FliS	122	4.9
		FliT	135	4.7
	EPEC/EHEC	CesAB	107	Alkaline
		CesA2	92	5.5

EspD) (Wainwright and Kaper, 1998), CesD2 (EspD) (Neves *et al.*, 2003a), CesAB (EspA and EspB) (Creasey *et al.*, 2003c) and CesA2 (EspA) (Su *et al.*, 2008).

Although these chaperone proteins involved in T3S are responsible for different substrates, they can be classified into 3 groups: Class I (effector proteins), Class II (the translocation pore) and Class III (The extracellular, helical components) (Table 1.4). Genes encoding T3 chaperones are normally carried by the pathogenicity island that also contains the genes encoding the other T3SS components. Although these chaperones share little sequence homology they are well conserved in protein structure (Fig. 1.7.) and have several widely known characteristics, such as a small size, an acidic pI (4-5) (Table 1.4), and a predicted amphiphilic α helix in the carboxy-terminal region. Class I chaperones bind their cognate effector

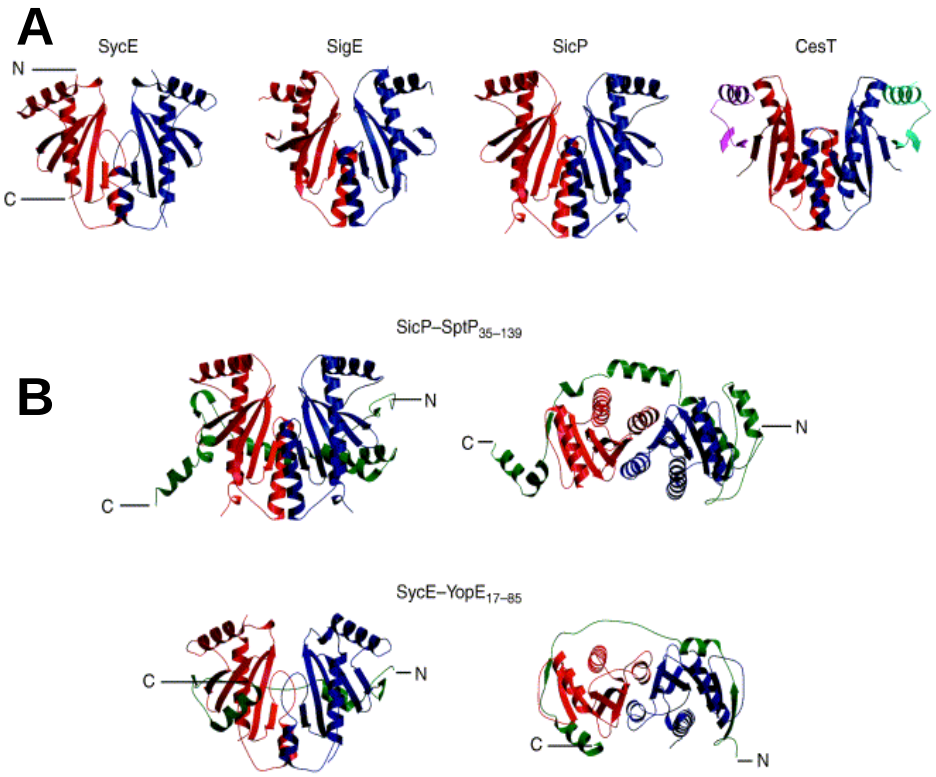


Fig. 1.7. A. Ribbon diagrams of SycE, SigE, SicP and CesT dimers; monomers are shown in blue and red. In the CesT dimer, the amino- and carboxy-terminal regions of each monomer (in magenta and cyan) are donated from adjacent molecules in the crystal. B. Ribbon diagrams of SicP–SptP35–139 and SycE–YopE17–85 complexes; both SptP35–139 and YopE17–85 are shown in green. One view is the same orientation as in (A), and the other has been rotated by 90° around the horizontal axis. Wild-type SptP and YopE have 543 and 219 residues, respectively. (Reproduced from Parsot *et al.*, 2003)

within their first 100 amino acids downstream of the N-terminal secretion signals and have two sub-categories which are chaperones associated with only one (class IB) or multiple (class IA) secreted virulence factors. Apparently, in EHEC, CesF which interacts with EspF only belongs to class IB and CesT which binds several effectors belongs to class IA. Crystallographic structures of the chaperones SicP and SigE from *Salmonella enterica*, SycE from *Yersinia pseudotuberculosis*, and CesT from

EPEC/EHEC/CR confirmed that class IA chaperones function as dimers (Birtalan and Ghosh, 2001, Luo *et al.*, 2001, Stebbins and Galan, 2001) (Fig. 1.7.).

As described in previous reports, CesT was known to interact with at least four T3 effector proteins (Tir, Map, EspF and NleA) in EHEC/EPEC/CR (Thomas *et al.*, 2005, Creasey *et al.*, 2003a, Abe *et al.*, 1999, Elliott *et al.*, 1999a, Creasey *et al.*, 2003b). Moreover, a very recent study demonstrated that this multi-effector T3 chaperone protein has five other binding partners (EspG, EspZ, NleG, NleH, and NleH2) (Thomas *et al.*, 2007) which were all discovered using a *sepD* deletion genetic background. CesT has been proven to be multi-functional (Elliott *et al.*, 1999a), therefore it is the most important and interesting chaperone protein among AE pathogens. Functionally, CesT is important for Tir expression as well as the secretion of multiple effector proteins (Thomas *et al.*, 2007, Li *et al.*, 2006, Thomas *et al.*, 2005, Creasey *et al.*, 2003a).

As it was first identified as a Tir chaperone protein, the interaction between CesT and Tir has been investigated in detail. Gel filtration studies showed that Tir-CesT forms a large multimeric complex (Elliott *et al.*, 1999a). It was revealed that CesT is not just responsible for Tir secretion or translocation via T3SS, but was also required for stabilizing Tir in the EHEC/EPEC cytoplasm (Abe *et al.*, 1999, Elliott *et al.*, 1999a). Further studies revealed that the amphipathic alpha-helical region of the C-terminal CesT was able to bind Tir, whereas the N-terminal residue of CesT was required for CesT

dimerization and translocation of Tir (Delahay *et al.*, 2002, Thomas *et al.*, 2005). Also the interaction between Tir-CesT complex and T3SS ATPase EscN was detected (Gauthier and Finlay, 2003) and EscN is required for T3 basal apparatus targeting (Inner membrane) of both Tir and CesT (Thomas *et al.*, 2005).

EspB and EspD translocators form a pore in the host membrane which is important for T3 virulence factor delivery. In order to open a channel between bacterium and host cell, there were specific chaperone proteins (Class II) involved in stabilising pore forming translocators in the bacterial cytoplasm and targeting translocon proteins for secretion. In EHEC/EPEC T3SS, it was known that CesD is critical for EspB and EspD secretion and a second chaperone protein CesD2 is also required for EspD export. (Wainwright and Kaper, 1998, Neves *et al.*, 2003a). Class II chaperones were proposed to be structurally different from the class I chaperones CesT/SigE (Pallen *et al.*, 2003). Pallen *et al.* detected tetratricopeptide-like repeats in the class II CesD/SycD/LcrH chaperone family but not in the class I CesT/SigE chaperone family that implied the functional diversity of two chaperone groups (Pallen *et al.*, 2003).

The class III chaperones were originally known as flagellar chaperones. Flagellar system used cytoplasmic chaperones for hook, cap or flagellin protein export (Aldridge *et al.*, 2003, Parsot *et al.*, 2003). Normally the chaperone binds to the C-terminal part of the substrate subunit and prevents subunit self-aggregation/interaction (Auvray *et al.*, 2001, Fraser *et al.*, 1999). In A/E lesion forming pathogens, EHEC/EPEC/CR, there were two chaperones (CesAB and CesA2) reported for EspA filament export (Creasey

et al., 2003c, Su *et al.*, 2008). CesAB and CesA2 are required for stabilising EspA protein in bacterium cell, while CesAB is only found critical for EspB secretion but not for the stability of intracellular EspB (Creasey *et al.*, 2003c, Su *et al.*, 2008). Also CesAB was found purified with the EspA monomer which suggested CesAB-EspA interactions prevent EspA aggregation (Creasey *et al.*, 2003c). Although CesAB shares several characteristics such as small molecular weight and α-helical structure, it has an alkaline pI which is uncommon (Creasey *et al.*, 2003c, Jackson *et al.*, 1998)

Chaperone-substrate interactions have been studied with different approaches (Wattiau *et al.*, 1994, Schesser *et al.*, 1996, Day and Plano, 1998, Hueck, 1998, Elliott *et al.*, 1999a, Neyt and Cornelis, 1999, Cheng and Schneewind, 1999, Aldridge and Hughes, 2001, Birtalan and Ghosh, 2001, Luo *et al.*, 2001, Stebbins and Galan, 2001). Although some of them still need further investigations, current observations suggest that these interactions prevented aggregation, limit toxic effects of misallocation of substrate and keep the substrate soluble within the bacterium before it is exported by the T3SS (Edqvist *et al.*, 2006, Letzelter *et al.*, 2006, Wilharm *et al.*, 2007, Johnson *et al.*, 2007, Paul *et al.*, 2008, Buttner *et al.*, 2008, Miki *et al.*, 2009, Hu *et al.*, 2009, Spaeth *et al.*, 2009, Cornelis, 2006). These chaperone escorting activities seemed very important in T3SS and chaperone recycling might also be happening in a very efficient way (Lee and Galan, 2004, Thomas *et al.*, 2005, Yip *et al.*, 2005a, Gonzalez-Pedrajo *et al.*, 2006, Johnson *et al.*, 2007, Thomas *et al.*, 2004, Evans *et al.*, 2006). In brief, chaperone proteins were required not only for facilitating T3 substrate secretion but also for substrate targeting selection/hierarchy in T3SS.

1.4.3.6 Substrate switching components of T3SS

Although the filamentous extension of the T3SS shows variation in length (Daniell *et al.*, 2001), the length of T3SS needle seems to be well controlled (Sekiya *et al.*, 2001). A similar regulation was also observed for flagellar secretion with the flagellar hook being normally 55nm and the length of FliC flaments being variable (Karlinsey *et al.*, 2000b, Minamino and Macnab, 2000). YscP is a key regulator for T3SS needle length in *Yersinia* spp. and FliK is responsible for hook length in the flagellar T3SS (Wagner *et al.*, 2009, Shibata *et al.*, 2007). There were different models presented which might explain how the needle length is controlled. It was hypothesised that YscP can act as an internal molecular ruler to measure needle length (Journet *et al.*, 2003). The loss of YscP resulted in various lengths of needle structure in *Yersina* and the length of the T3SS needle is dependent on the length of an extended YscP (Journet *et al.*, 2003). This theory was tested by swapping two different size YscP proteins.; when cross-complementary experiments were performed in *Yersinia* spp., the shorter YscP (*Y. pestis*) also produced shorter YscF needles which suggested the YscF needle was controlled by the actual size of YscP (Journet *et al.*, 2003). Another 'cap model' was proposed in which flagellar hook length is determined by the amount of subunit protein secreted by the flagellar apparatus (Makishima *et al.*, 2001). However the exact mechanism underlying this model is not clear and two recent studies suggested that FliK can also act as an internal ruler, as discussed for YscP, which favours the ruler model of needle/hook length control (Shibata *et al.*, 2007, Erhardt *et al.*, 2010). In addition, a molecular clock model has been suggested which implies that the timing is critical for the needle length as well as a FliK ruler (Moriya *et al.*, 2006). In both

Salmonella and *Shigella*, a mechanism has been proposed in which the completion of the inner rod determines the needle length and substrate switching (Marlovits *et al.*, 2004). Ruler proteins, YscP and FliK, have a reserved C-terminus responsible for substrate switching and has been termed the 'T3S4' domain, standing for Type 3 secretion substrate specificity switching (Agrain *et al.*, 2005, Minamino and Macnab, 2000, Minamino *et al.*, 2004). This T3S4 domain switches substrate specificity to effector secretion and the ruler is then released. There was another proposed key molecule for substrate switching, YscU (*Yersinia* T3SS) or FlhB (Flagellar T3SS). YscU, as well as FlhB, was known as a self-cleavable protein (a 30-kDa N-terminal and a 10-kDa C-terminal part) required for translocator export (Lavander *et al.*, 2002, Sorg *et al.*, 2007). YscP export was reduced in a non-cleavable YscU mutant and therefore result in longer YscF needle produced and no translocator exported (Sorg *et al.*, 2007). So it suggested that self –cleavage of YscU is required for translocator recognition and YscP export which controls YscF needle length (Lavander *et al.*, 2002, Sorg *et al.*, 2007, Riordan and Schneewind, 2008). In the flagellar system, FlhB was shown to interact with FliK to work as a sensor and pass a signal relating to hook completion to self-cleavable FlhB for substrate switching (Minamino *et al.*, 2004, Ferris *et al.*, 2005). This is very similar to what is proposed to happen in the regulation of *Yersinia* T3SS. In EPEC/EHEC, EscU and EscP were proposed to control this key step as homologues of YscU and YscP respectively (Zarivach *et al.*, 2008, Pallen *et al.*, 2005a) and further investigation is required to reveal more details of this mechanism in EHEC/EPEC.

1.4.3.7 The locus of enterocyte effacement encoded regulator (Ler)

As with other complex biological systems, T3SS is controlled by many regulators in A/E pathogens. T3SS expression is regulated by various factors encoded both within and outside the LEE (Bustamante *et al.*, 2001, Sanchez-SanMartin *et al.*, 2001, Schauder *et al.*, 2001, Goldberg *et al.*, 2001, Umanski *et al.*, 2002, Sperandio *et al.*, 2002, Haack *et al.*, 2003, Lio and Syu, 2004, Sharma and Zuerner, 2004, Iyoda and Watanabe, 2004, Tsai *et al.*, 2006, Dong and Schellhorn, 2009b, Shakhnovich *et al.*, 2009, Hansen and Kaper, 2009, Bhatt *et al.*, 2009). H-NS (histone-like nucleoid-structuring protein) is a global DNA-binding transcriptional dual regulator which directly or indirectly alters the expression of 5% of all genes and 69% of the temperature regulated genes in *Escherichia coli* strain K12 (White-Ziegler and Davis, 2009, Bertin *et al.*, 2001, Hommais *et al.*, 2001). In A/E lesion forming pathogens, H-NS is a repressor of LEE expression in a temperature-dependent manner by directly binding to their promoters or the regions nearby (Umanski *et al.*, 2002). However, The locus of enterocyte effacement encoded regulator (Ler) (Fig.1.8), acting as an anti-repressor, can antagonize the H-NS-dependent inhibition of LEE expression (Bustamante *et al.*, 2001) by interfering with H-NS binding. On the other hand, it has been suggested that Ler acts as a LEE5 activator in an H-NS-independent manner (Umanski *et al.*, 2002). It is not just important for LEE expression, but also proved crucial for expression of certain non-LEE T3S-associated factors (Elliott *et al.*, 2000, Roe *et al.*, 2007). Ler is therefore an important and general regulator/activator of T3S in A/E pathogens. Although other signalling pathways have been found to be Ler-independent that regulate T3SS in A/E

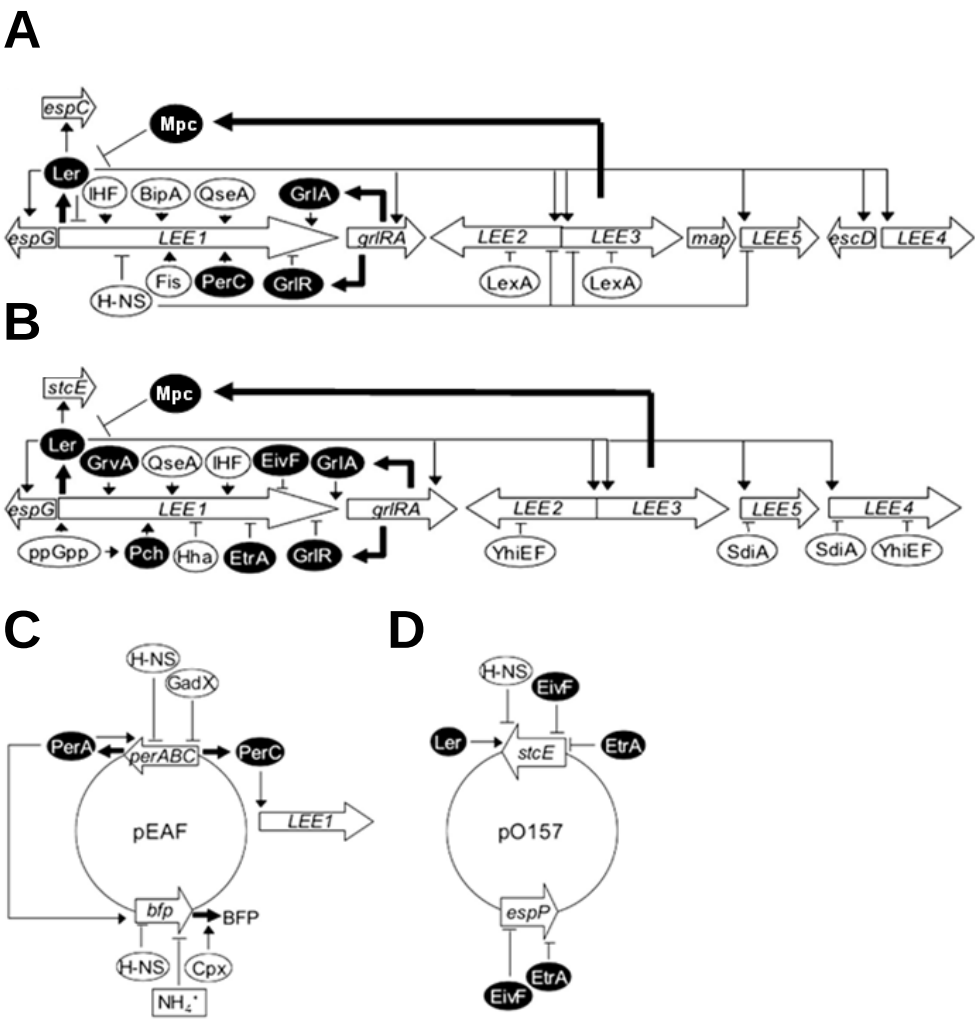


Fig. 1.8. Regulation of the LEE PAI in EPEC (A) and EHEC (B). Virulence gene regulation in the EAF plasmid of EPEC (C) and the EHEC pO157 plasmid (D). Thin arrows represent positive regulatory signals, and thin blunt arrows represent negative signals. Solid arrows indicate expression of regulatory proteins. Horizontally acquired regulatory proteins appear in ovals with a black background, and regulatory proteins endogenous to *E. coli* are within ovals with a white background. (Modified from Mellies *et al.*, 2007)

Escherichia coli, the majority of regulatory mechanisms were demonstrated to be mediated via Ler.

Ler is encoded by the first gene of LEE1 operon and the expression of Ler is controlled by many factors by binding to its promoter or upstream regions. For example, GrlA, a LEE-encoded activator, was shown to stimulate Ler

expression via direct binding to the promoter region of the *LEE*1 operon (Huang and Syu, 2008). However, GrlR, a LEE repressor, negatively regulates the expression of LEE via sequestering GrlA (Barba *et al.*, 2005, Jobichen *et al.*, 2007, Huang and Syu, 2008). PerC (plasmid-encoded regulator C) was found important for LEE expression via a Ler-dependent manner in EPEC (Mellies *et al.*, 1999). However, the PerC regulator is not present in EHEC but has several chromosomal homologues instead (Iyoda and Watanabe, 2004). These Pch proteins activate LEE1 transcription and bind to its promoter region directly (Abe *et al.*, 2008). Quorum sensing (QS) in EHEC was also demonstrated to activate LEE expression with QseA, a key QS factor, up-regulating the transcription of LEE1 via directly binding upstream of the LEE1 promoter region (Sharp and Sperandio, 2007). Ler itself, could act as an autoregulator and at high levels can repress LEE1 expression by binding to its own promoter region (Berdichevsky *et al.*, 2005). Besides the regulation pathways via Ler expression, another kind of pathway is also implied by other studies in A/E pathogens. A newly identified regulator Mpc (multiple point controller) was known to interact with Ler and suppresses the expression of the LEE proteins (Tsai *et al.*, 2006). Another very recent publication indicates that Mcp might be a chaperone protein for SepL, therefore suggesting it be renamed as CesL. However the function of Mpc/CesL requires more intensive investigation in future experiments (Younis *et al.*).

1.5 Previous work leading to this project

LEE4 encodes the secreted proteins EspA,D,B as well as other factors, such as SepL and EscF. EspA,D,B have been shown to be necessary for A/E

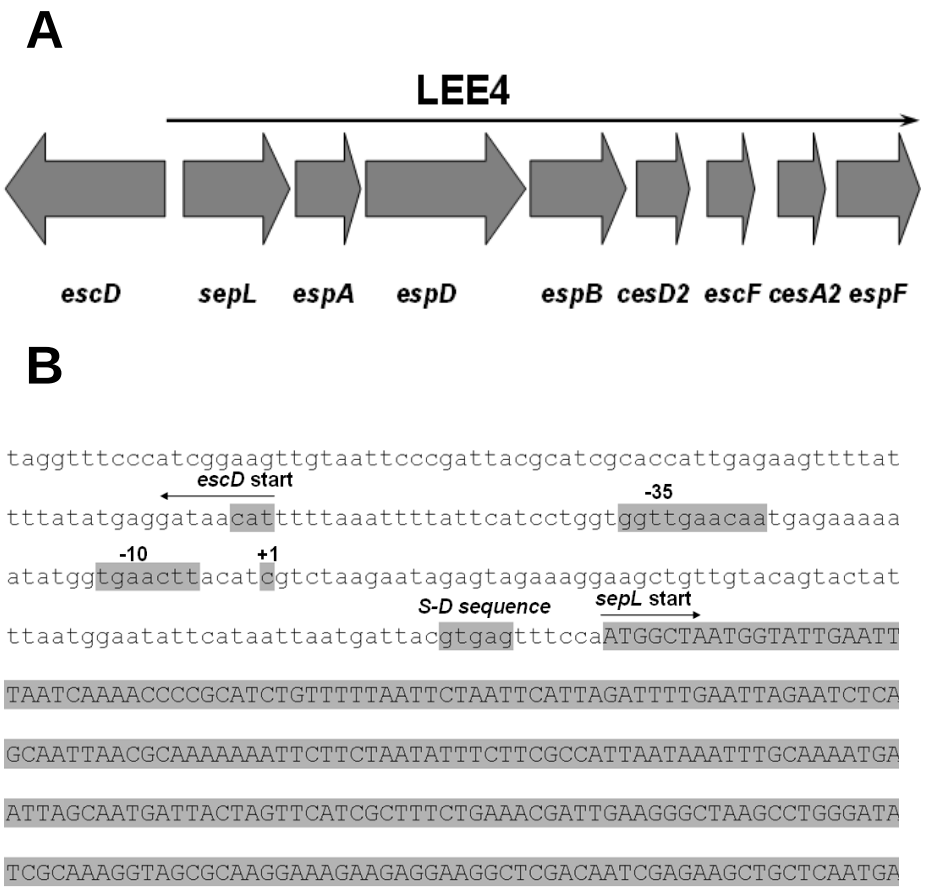


Fig. 1.9. (A). Genes located within LEE4 Operon; (B) Nucleotide sequence of the DNA fragment containing the *sepL* promoter and part of its open reading frame (ORF). The start of transcription (+1), the 10 and 35 consensus sequences, the Shine-Dalgarno (SD) sequence and *sepL* ORF are shaded as described in (Kresse *et al.*, 2000).

lesion formation and signalling within host cells as translocator proteins. Formation of this EspA filament structure is transient and disappears once attachment is strengthened by intimate attachment (Ebel *et al.*, 1998, Knutton *et al.*, 1998). So, the expression and secretion of EspA and EspB potentially happens early during infection and are enhanced when bacteria are grown at 37°C in tissue culture medium and by the presence of micronutrients or signals produced by eukaryotic cells (Ebel *et al.*, 1996, Jarvis and Kaper, 1996). Although *sepL* and *espADB* genes all locate within the same LEE4 operon, the transcription levels of these genes are different

(Beltrametti *et al.*, 1999, Roe *et al.*, 2003b). At least two putative promoters for *espADB* were revealed by previous work by primer extension (Beltrametti *et al.*, 1999, Mellies *et al.*, 1999). However the theory of two promoters is debatable. The work in our laboratory showed no evidence of any expression from a promoter in front of *espA*, with expression only detectable from *sepL* (Roe *et al.*, 2003b). An analysis of LEE4 mRNA levels and EspD levels in whole cells confirmed that the abundant transcript was not being translated to give an intracellular pool of Esps in low-secretor strains (Roe *et al.*, 2003b), indicating significant translational control of the transcript. Further data from our laboratory supported posttranscriptional control of translocator expression, although the mechanism still remains to be identified. Previous work demonstrated that SepL is a key protein controlling T3 secretion and no further information on SepL expression was available (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Deng *et al.*, 2005, Deng *et al.*, 2004). Therefore, it would be valuable to investigate the expression regulation of SepL for understanding T3SS in EHEC O157.

sepL is the first gene on the LEE4 operon which is about 5.6kb and followed by *espADB* genes (Beltrametti *et al.*, 1999) (Fig.1.9E). The most well known regulator of EHEC T3SS is Ler which was shown activating *espADB* genes transcription (Sharma and Zuerner, 2004). It was known that *sepL* and *espADB* were co-transcribed and therefore Ler might also have an impact on *sepL* transcript. From previous studies, it was suggested that the *espADB* transcript is processed in a post transcriptional manner (Roe *et al.*, 2003b). Its expression was sigma S dependent and controlled by multiple factors which included temperature, cell contact and ion concentration (Beltrametti *et al.*, 1999, Creasey *et al.*, 2003c, Ide *et al.*, 2003, Roe *et al.*, 2003b, Deng

et al., 2005). The SepL protein has been demonstrated to be important for controlling the secretion of both translocon and effector proteins (Deng *et al.*, 2005, Deng *et al.*, 2004, Kresse *et al.*, 2000, O'Connell *et al.*, 2004). Pallen *et al.* revealed the N-terminal sequence (267 aa) of SepL is homologous to YopN and the C-terminal sequence (83 aa) is homologous to TyeA (Pallen *et al.*, 2005a). The YopN-TyeA family includes YopN-TyeA (*Yersinia* spp.), SepL (EHEC/EPEC/CR), SsaL, InvE (*Salmonella* spp.), MxiC (*Shigella* spp.) and PopN (*Pseudomonas aeruginosa*) (Pallen *et al.*, 2005a). It was reported that YopN, MxiC and PopN can be secreted via T3SS while no evidence suggested SepL, SsaL and InvE can be exported so far (Yang *et al.*, 2007, Botteaux *et al.*, 2009, Kubori and Galan, 2002, Forsberg *et al.*, 1991, Yu *et al.*, 2010, Kresse *et al.*, 2000). YopN was suggested acting as a key protein to switch on effector secretion in *Yersinia* spp. and a similar role for PopN in *Pseudomonas aeruginosa* (Cornelis and Wolf-Watz, 1997, Yang *et al.*, 2007). MxiC was recently shown to be required for effector secretion (Botteaux *et al.*, 2009). InvE, as well as SsaL, was proposed controlling the secretion of translocators but not effector proteins export (Kubori and Galan, 2002, Coombes *et al.*, 2004). Although SepL has been shown important for translocator export, a SepL deficent EHEC/EPEC also exhibits hypersecretion of effector proteins (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Deng *et al.*, 2005). So far, all the published work has mainly focused on the functional importance of SepL. In this thesis I have also concentrated on SepL expression and the link to the post transcriptional regulation of LEE4.

So far, SepL and its homologues have demonstrated strong impact on T3 secretion. As a binding partner of SepL, SepD was also investigated

previously and a Δ*sepD* strain exhibited a similar phenotype as a sepL mutant (Deng *et al.*, 2004, Deng *et al.*, 2005). This evidence suggests that SepL and SepD may play a role as a molecular switch controlling secretion of translocators and effectors. SepL and SepD are demonstrated interacting with each other and both proteins are shown bacterial membrane associated in EHEC (Deng *et al.*, 2005). However, the precise mechanism of SepL-SepD controlling the secretion switch between translocons and effectors was unknown. In my study, SepL was investigated functionally according to several properties of this protein.

1.6 Aims of the work

1.6.1 An analysis of SepL expression and localization

The first key aim of the research is to localise SepL and SepD in comparison with the T3S apparatus and EspA filaments in single bacterial cells. SepL will be fused to fluorescence protein reporters and visualized by fluorescence microscopy. SepL and SepD fusions to GFP will be localized by Western blotting of bacterial fractions. Environmental and genetic factors that alter the localisation will be studied including mutations of defined T3S proteins.

1.6.2 Translation and processing of the *sepL* mRNA transcript

The second key aim of the research is to determine if SepL production is heterogeneous and how the LEE4 transcript is processed and translation regulated to generate heterogeneity. From previous data, there is evidence that the expression of SepL is controlled at a post-transcriptional level. The

aim is to verify then understand this post-transcriptional regulation. *sepL* and LEE4 sequences important for this post-transcriptional regulation will be determined by mutational analysis.

1.6.3 The interaction and function of SepL and SepD

The third aim of the research is to attempt to identify binding partners of SepL and SepD associated with the EHEC T3SS. SepL and SepD are crucial for the secretion of EspADB but not effector proteins. The mechanism has not been elucidated so far. Here, the aim is to define functional regions of the SepL protein. The research aims to determine if SepL/SepD proteins can bind to other T3 related targets. In particular, SepD and SepL will be over-expressed and their interactions studied with biochemical techniques.

Chapter2

Regulation of SepL expression

2.1 Introduction

SepL has been shown to be critical for the control of T3S, in particular the switch between translocator protein (EspADB) and effector protein secretion (Deng *et al.*, 2004, O'Connell *et al.*, 2004, Deng *et al.*, 2005). The balance of this secretion is also controlled by environmental conditions. From previous studies, it is established EspADB cannot be secreted in a *sepL* mutant and therefore no EspA filaments are formed on the bacterial surface (Kresse *et al.*, 2000). Several published works have also shown that EspADB secretion is required for effector translocation (Knutton *et al.*, 1998). For example, Tir translocation is completely blocked by deleting *espA* and this can be complemented by bringing back *espA in trans*.

There have been three studies examining *sepL* mutants published by Kresse *et al,* Deng *et al* and O'Connell *et al.* These mutants were generated by inserting or deleting a partial fragment in the middle of *sepL* which takes *sepL* out of frame and all of these mutations could be complemented by *sepL* provided *in trans*. Work within our own group has indicated that EspA filament expression is under post transcriptional control and that production of EspA filaments within a population is heterogeneous (Roe *et al.*, 2003b, Roe *et al.*, 2004). Certain strains have a relatively high proportion of the population producing EspA filaments under conditions known to induce T3S, whereas the majority of strains examined only had a minor subset of the population expressing EspA filaments. As *sepL* is the first gene in the LEE4 operon that also contains *espADB*, I wanted to know more about the regulation of *sepL* and whether it is subject to post-transcriptional control. As SepL is essential for EspA filamentation, it was hypothesized that the key

checkpoint in translocon expression that is responsible for the heterogeneity will be the transcription and translation of SepL. In this study, therefore, several *sepL* mutants and fusions were constructed and analysed to examine SepL expression.

It has been shown that T3SS expression is controlled at a post transcriptional level (Chilcott and Hughes, 2000, Roe *et al.*, 2003b). Hfq (host factor required for phage Qβ RNA replication), which also know as HF-I (host factor I), was originally identified in *Escherichia coli* as an essential bacterial protein for RNA replication of bacteriophage Qβ in 1968 (Franze de Fernandez *et al.*, 1968, Shapiro *et al.*, 1968). It was found to be present in both Gram-positive and Gram-negative bacteria using phylogenetic analyses (Sun *et al.*, 2002). *Escherichia coli* Hfq is a very abundant small protein which normally forms hexamers and previous studies indicated a homology link between Hfq and spliceosomal Sm proteins (Sauter *et al.*, 2003). Many reports have suggested that Hfq can act as a post-transcriptional regulator via its ability to bind to RNA (Valentin-Hansen *et al.*, 2004, Nogueira and Springer, 2000, Urban and Vogel, 2007, Urban and Vogel, 2008, Papenfort *et al.*, 2009, Shakhnovich *et al.*, 2009). Hfq acts as a sRNA chaperone which can bind to non-encoding small RNA (sRNA) and protect it from degradation (Lease and Belfort, 2000, Moll *et al.*, 2003b, Storz *et al.*, 2004). Specific sRNAs can bind to specific mRNA transcripts and control gene expression (Vogel and Sharma, 2005, Vogel and Wagner, 2007). For example, an sRNA-mRNA complex is able to alter a structure normally inhibiting translation and therefore enable ribosome access to the SD sequence and AUG start codon (Moll *et al.*, 2003b). In other cases, an

sRNA/mRNA interaction can block ribosome access to target mRNA (Moll *et al.*, 2003b). Further studies suggested that Hfq binding is critical for stabilisation of sRNA – mRNA complex by changing its secondary structure (Moll *et al.*, 2003b). Therefore this Hfq/sRNA binding to mRNA can result in altered structures/presentation of target mRNAs. For some sRNA, such as DsrA, Hfq appears not to change its secondary structure and it is more likely to increase the concentration of the sRNA-mRNA complex by stabilizing the interaction (Brescia *et al.*, 2003). By either mechanism, Hfq can modulate sRNA–mRNA complexes and regulate target gene expression post-transcriptionally. From previous studies, it was known that Hfq has two RNA binding sites: the proximal site binds to sRNA and AU rich regions of mRNA (Sonnleitner *et al.*, 2004); another distal site binds to poly (A) tails (Folichon *et al.*, 2003). Apart from those RNA interactions, Hfq interacts with PAP I, PNPase and RnaseE which are involved in RNA degradation (Viegas *et al.*, 2007, Mohanty *et al.*, 2004). Taken together, this information implies that Hfq is a key protein involved in post-transcriptional regulation.

Although Hfq was originally identified as an RNA regulator, it was shown to have an important role in modulating virulence expression together with certain sRNAs in bacterial pathogens (Chao and Vogel, 2010 Muffler *et al.*, 1996, Nogueira and Springer, 2000, Sledjeski *et al.*, 2001). More and more evidence shows that small non-coding RNAs are involved with virulence-related expression in pathogens with T3SSs (Vogel and Papenfort, 2006, Vogel and Wagner, 2007). The key component of the carbon storage regulator (Csr) and its homologous repressor of secondary metabolites (Rsm) systems is an RNA binding protein (CsrA or RsmA) that regulates gene expression post-transcriptionally by changing target mRNA stability and/or

interfering with ribosome binding (Romeo, 1996, Romeo, 1998, Majdalani *et al.*, 2005). The Csr system participates in global regulation affecting central carbon flux, motility, biofilm formation, quorum sensing and extracellular factor production (Romeo, 1998, Nogueira and Springer, 2000, Jackson *et al.*, 2002, Majdalani *et al.*, 2005, Viegas *et al.*, 2007, Vogel, 2009). For example, CsrA acts as an activator of FlhDC expression (Wei *et al.*, 2001, Jackson *et al.*, 2002). It was also reported that the Csr system was found essential for bacterial pathogenesis and successful infection in the animal host probably by controlling the different physiological stages of pathogen infection (Lucchetti-Miganeh *et al.*, 2008). CsrA activity was known to be sequestrated via binding to its small non-coding RNA antagonists, CsrB and CsrC (Babitzke and Romeo, 2007). Previous studies suggested that CsrA bound to a "CAGGXXG" sequence with higher affinity and CsrB and CsrC both have multiple conserved CsrA binding motifs (Babitzke and Romeo, 2007, Majdalani *et al.*, 2005). A recent study revealed that CsrB and CsrC degradation is linked to the RNaseE degradosome (Suzuki *et al.*, 2006). Since Hfq also interacts with RNaseE and PNPase, two key components of the RNA degradsome, it implies that Hfq can also mediate the Csr system via CsrBC RNA degradation (Basineni *et al.*, 2009, Mohanty *et al.*, 2004). According to this information, *hfq* and *csrA* were logical targets to delete and investigate their impact on the post-transcriptional control of LEE4.

2.2 Results

2.2.1 Characterization of SepL and SepD mutants

SepL has several predicted functional domains (Kresse *et al.*, 2000) but the functions of these domains are still unknown. In order to further investigate both the action and expression of SepL in EHEC O157:H7, various *sepL* mutants were constructed using allelic exchange with a temperature-sensitive plasmid─pIB307 as described in Materials and Methods. There were 5 different *sepL* mutants generated using the same allelic exchange strategy (Fig. 2.1A) Western blotting to examine the impact of the *sepL* mutations on T3 translocon expression and secretion was carried out using an EspD antibody in Western blotting assays. At the time of this work, a total deletion of the *sepL* open reading frame has not been published so it is interesting to test the impact of a total *sepL* deletion on T3SS in EHEC. As will become clear through this chapter, differences in *sepL* mutants are likely to be relevant as they will result in different final LEE4 mRNA transcripts and so both the impact on SepL production and the *sepL-espADB* mRNA need to be considered. In this study, a *sepL* total knockout mutant (ZAP1143) shows a typical *sepL*/*sepD* deletion phenotype when cultured in MEM-Hepes medium. It has been established across a number of studies that this medium promotes T3S by EHEC O157 strains. It was noted that the complete *sepL* deletion also impairs EspD production as less EspD was detected in the whole cell fraction. Interestingly, this phenotype was not complemented by plasmid-based *sepL* expression (Fig. 2.1B; left and middle panel). As stated, this irreversible phenotype might be due to a change in the LEE4 mRNA structure caused by deleting *sepL*. In order to make the complete deletion, the *sepL* gene is replaced by a *sacB-kana*^{R} cassette. For

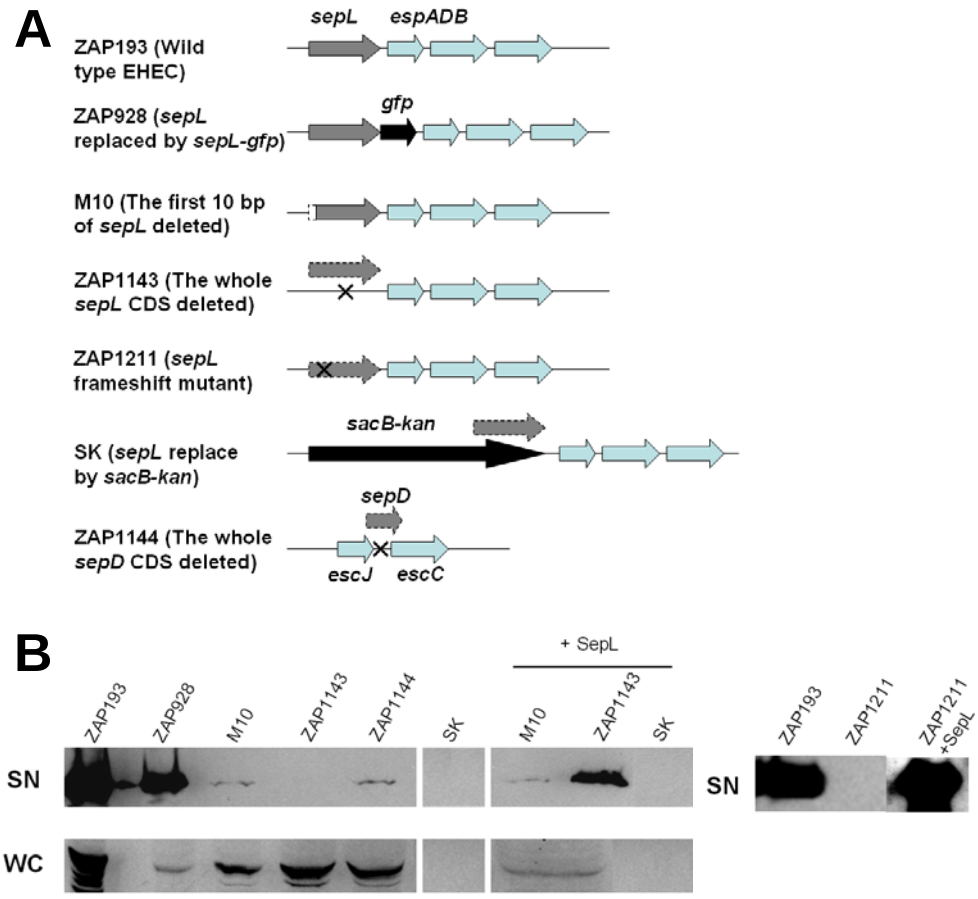


Fig. 2.1. Characterization of *sepL* and *sepD* mutants.
Different strains (A) were cultured in MEM-Hepes and supernatant (SN) and whole cell (WC) samples were prepared as described in Materials and Methods. EspD was detected with an anti-EspD antibody (B).

this strain, EspD expression is totally blocked and it cannot be restored by *sepL in trans*. This insertion is very likely to prevent downstream *espADB* expression from LEE4 operon. A third construct produced was a chromosomally integrated *sepL-gfp* strain (ZAP928) to examine SepL localization. Unexpectedly, this replacement also caused a reduction in EspD expression. This could be a result of impaired SepL function as fused to GFP, but it may also be a result of polar effects on the *espADB* transcript as above in which deleting *sepL* prevented other LEE4 genes being transcribed. To test the significance of the mRNA structure on EspD production, another

sepL mutant (ZAP1211) was constructed which has a minimal impact on the LEE4 mRNA. In ZAP1211, the *sepL* CDS was taken out of frame by insertion of an additional base at nucleotide position 345 of *sepL*. When this mutated strain was examined for T3S capacity, it also shows a typical *sepL*/*sepD* deletion phenotype and could be fully complemented by plasmid based *sepL* expression (Fig. 2.1B; right panel) unlike the other *sepL* constructs described above.

2.2.2 Heterogeneous expression of SepL-GFP

Previously research had indicated that the *espADB* transcript was subject to post-transcriptional control leading to heterogeneous surface expression of EspA filaments in EHEC O157 strains (Roe *et al.*, 2003b). *sepL* is the first gene on the LEE4 operon followed by the *espADB* genes and a *sepL* mutant failed to secrete EspADB translocon proteins into the culture supernatant (Kresse *et al.*, 2000). From previous studies, it is evident that *sepL* is initially expressed as part of a *sepL-espADB* transcript that is subsequently processed (Roe *et al.*, 2003a, Roe *et al.*, 2003b). For this research, I wanted to determine if SepL is also regulated by the same mechanism and determine whether it also exhibits heterogeneous expression. To examine the expression of SepL in individual bacteria, the whole *sepL* ORF was amplified from EHEC O157, including its own promoter region, and fused to *gfp* in frame. This fusion was transformed into EHEC O157 strain ZAP193 (a high secretor strain) and ZAP108 (a low secretor strain) and SepL-GFP expression examined by fluorescence microscopy as described in Materials and Methods.

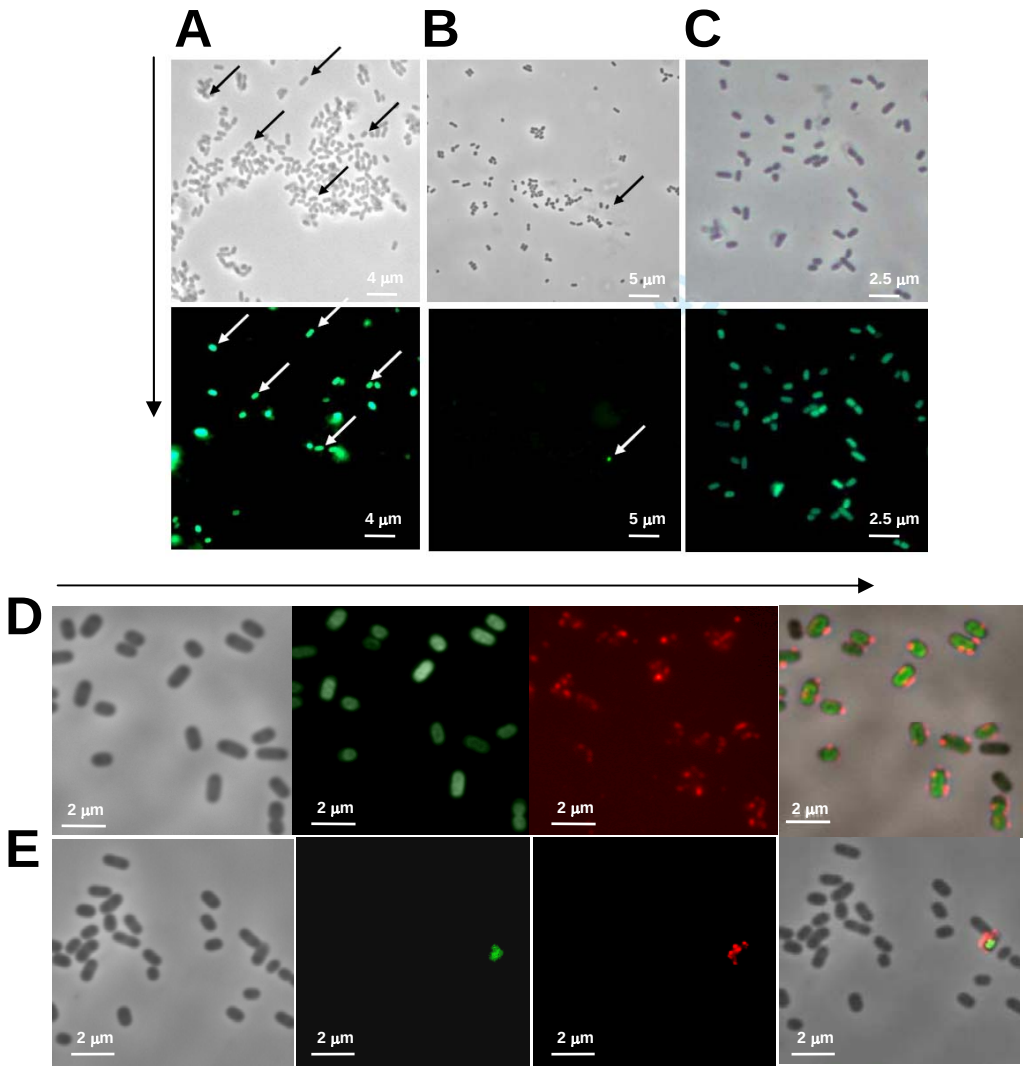


Fig. 2.2. Heterogeneous expression of SepL-GFP and correlation with EspA expression at the single cell level. (A) *E. coli* O157 (ZAP193) was transformed with pDW6 containing full length *sepL* fused to *gfp* expressed from the *sepL* promoter. Bacteria were cultured in MEM-Hepes to an OD600 of ~0.8 and the culture fixed for fluorescence microscopy. A subset of the bacteria express the fusion at a high and detectable level, examples are indicated by arrows, under the exposure conditions used (Chapter 5). (B) A low secretor strain, *E. coli* O157 ZAP108 (Roe *et al.*, 2003b) was also transformed with pDW6 and examined under the same conditions. In this field only one bacterium (highlighted by an arrow) expressed a high and detectable level of the SepL-GFP fusion. (C) By comparison, transformation of ZAP193 with an *rpsm-gfp* fusion results in expression of detectable GFP fluorescence in the majority of bacteria (>99%). (D) Correlation between SepL-GFP expression and EspA filament production on the bacterial cell surface. ZAP193 containing pDW6 was cultured and fixed as above with appropriate steps using primary and secondary antibodies to detect EspA filaments as described in Materials and Methods. A subset of bacteria did not express GFP fluorescence and these same bacteria (examples indicated by arrows) do not express EspA filaments on the surface. (E) To confirm the plasmid-based studies of *sepL-gfp* expression, the same construct was exchanged onto the chromosome to replace native *sepL*. This construct has a significantly altered *sepL-espADB* mRNA as a result of the introduction of *gfp* but still produced EspA filaments on a subset of cells and this correlated with GFP expression (indicated by the arrow).

The expression of SepL-GFP was only found in a subset of bacteria (Fig. 2.2.A-B). Under the same culture conditions, all bacteria expressed an *rpsM-gfp* fusion (Fig. 2.2.C) confirming that the proportional expression was not due to cell viability or issues specific to GFP. As our previous work has demonstrated that production of EspA filaments is also heterogeneous in EHEC O157, I used indirect immuno-fluorescence to detect EspA filaments on the strain containing the full length SepL-GFP fusion. Of those bacteria expressing EspA filaments, 91% had SepL-GFP expression level higher than 22.5 relative fluorescence units (rfu) (Fig. 2.2A), whereas only 9% of EspA-filamented bacteria had expression levels less than 22.5 rfu. Conversely, 82% of bacteria with no detectable surface EspA filaments had undetectable or low (<22.5 rfu) SepL-GFP expression. This data demonstrates a positive association between SepL-GFP translation and EspA filamentation as is evident in Fig. 2.2D. To rule out issues with copy number for the plasmid-based *sepL-gfp* construct, the same translational fusion was used to replace *sepL* on the chromosome (ZAP928). This strain still produced EspA filaments but at a reduced level compared to the parent strain. In this background SepL-GFP expression also correlated with EspA filamentation (Fig. 2.2E). It is therefore evident that SepL expression is also heterogeneous and may account for the checkpoint in EspADB translocon production as SepL is required for EspADB secretion.

2.2.3 Expression of SepL-GFP in different LEE genetic backgrounds

I next wanted to determine what factors control the expression of SepL. The expression of the full length SepL-GFP fusion (pDW6) was measured in different genetic backgrounds (Fig. 2.3 and 2.4). The first genetic

background tested was a *sepL* deletion strain. Total fluorescence measurement was carried out as described in Materials and Methods. There was no obvious difference in fluorescence level observed between the EHEC wild type and Δ*sepL* backgrounds (Fig. 2.3). From this it is implied that the chromosomal integrated *sepL* gene doesn't have an effect on the expression of SepL-GFP from the plasmid.

Expression of the fusion was reduced 12.5 fold (OD_{600nm} = 0.6) in a *ler* deletion background demonstrating that in addition to controlling LEE1/2/3/5 expression (Fig.2.4A). Ler also has a significant impact on *sepL*/LEE4 regulation. In a background in which *escRSTU* was deleted, preventing the assembly of the normal basal apparatus of the

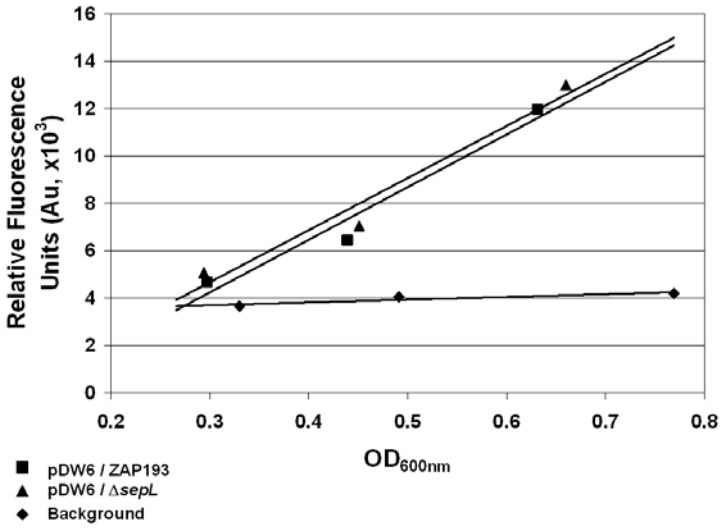


Fig. 2.3. SepL-GFP expression in EHEC O157 Δ*sepL* genetic backgrounds. SepL-GFP expression was determined in strains transformed with the *sepL-gfp* fusion (pDW6) cultured in MEM-Hepes. Fluorescence expression was carried out as described in Materials and Methods. (■) Wild type EHEC O157 ZAP193 (pDW6); (▲) Δ*sepL* ZAP1211 (pDW6); (◆) EHEC O157 ZAP 193 (control, no *gfp* plasmid). Each expression experiment was carried out on at least three occasions and one replicate is shown. Data cannot be combined from different experiments as the sampling optical density points are different each time

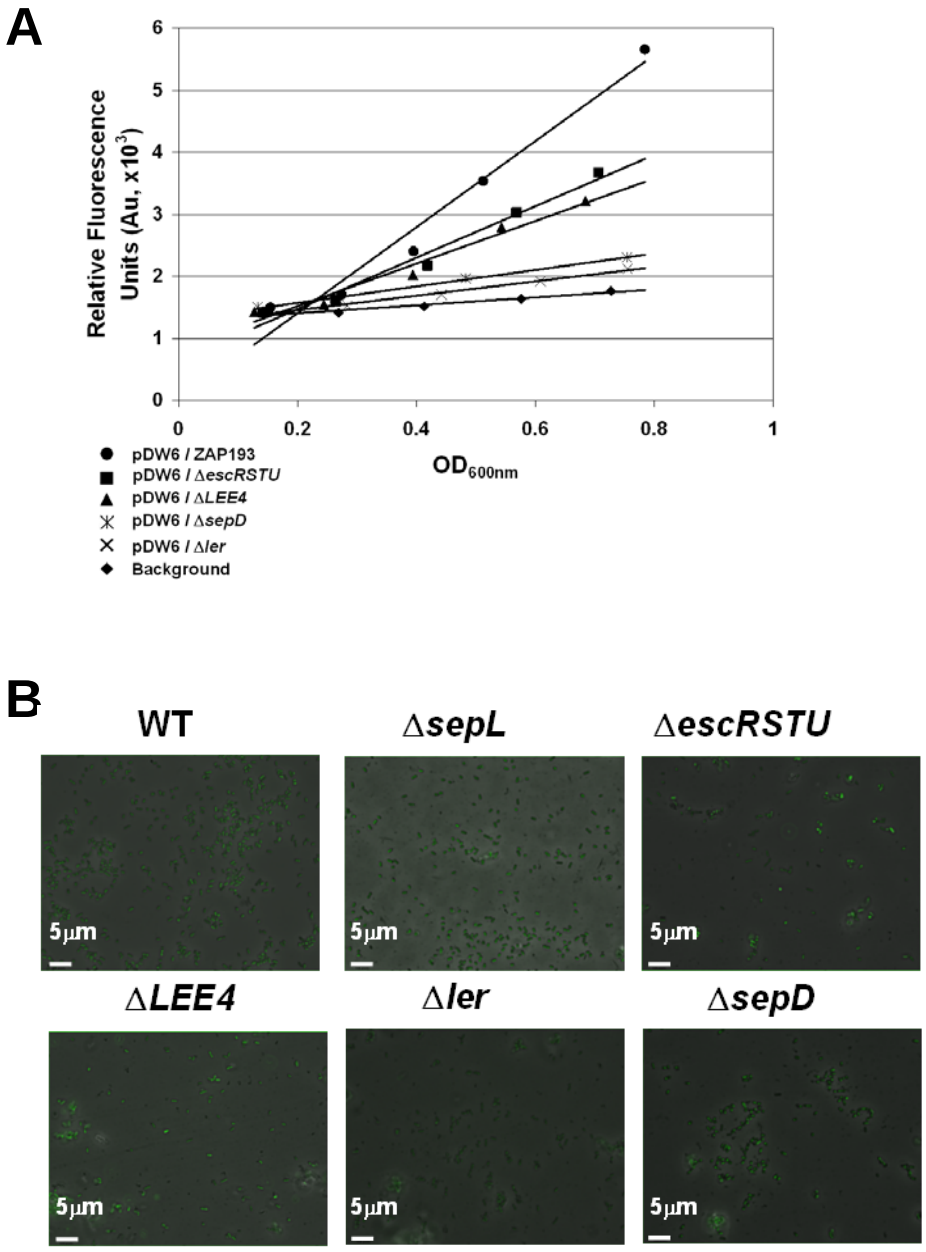


Fig. 2.4. SepL-GFP expression in different EHEC O157 genetic backgrounds. (A) SepL-GFP expression was determined in strains transformed with the *sepL-gfp* fusion (pDW6) cultured in MEM-Hepes. Fluorescence expression was carried out as described in Materials and Methods. (●) Wild type EHEC O157 ZAP193 (pDW6); (■) Δ*escRSTU* ZAP1140 (pDW6); (▲) Δ*LEE4* ZAP984 (pDW6); (✳) Δ*sepD* ZAP1144 (pDW6); (×) Δ*ler* ZAP1004 (pDW6) (◆) EHEC O157 ZAP 193 (control, no *gfp* plasmid). Each expression experiment was carried out on at least three occasions and one replicate is shown. Data cannot be combined from different experiments as the sampling optical density points are different each time. (B) SepL-GFP heterogenous expression in individual cells were examined under fluorescence microscope, no significant change of SepL-GFP heterogenous expression was observed in these genetic backgrounds.

T3SS, SepL expression at OD_{600nm} = 0.6 was reduced by 50%. A 55% reduction of SepL-GFP expression was detected in a LEE4 deletion background (Fig.2.4A). However, expression of SepL-GFP was reduced 6.7 fold (OD_{600nm} = 0.6) in a *sepD* deletion. The expression of SepL-GFP was then examined in the individual bacteria in the same backgrounds. As indicated by the low level of expression in those genetic backgrounds especially in a *ler* background, SepL-GFP was difficult to detect (Fig.2.4B). However, in my study, no evidence suggested that SepL heterogeneity was changed significantly in these mutants.

2.2.4 Tir expression is not affected by *sepL*/*sepD* deletion

In this study, SepL expression was demonstrated to be reduced in a *sepD* deletion background as shown in Fig. 2.4A. Previous work in our laboratory has already shown that there is co-ordinated single cell expression between LEE4 (*espA*) and LEE5 (*tir*) in EHEC O157:H7 strains. When EHEC O157 (ZAP193) was cultured in a T3S permissive condition, it was found that LEE4 and LEE5 expression was detected in the same subset of bacterial cells (Roe *et al.*, 2004). To test whether Tir expression was also regulated by SepL or SepD, a full length Tir-GFP translational fusion was made (pDW-tir) and transformed into different genetic backgrounds. Total fluorescence was measured as described in Materials and Methods. Although SepL expression was repressed in a *sepD* mutant, Tir expression was not altered in this background (Fig. 2.5). Similarly, Tir expression was not affected by deletion of *sepL* (Fig. 2.5). These results suggest that both SepL and SepD are not specifically involved in the co-ordinated heterogenous expression of LEE4 and LEE5 in EHEC O157.

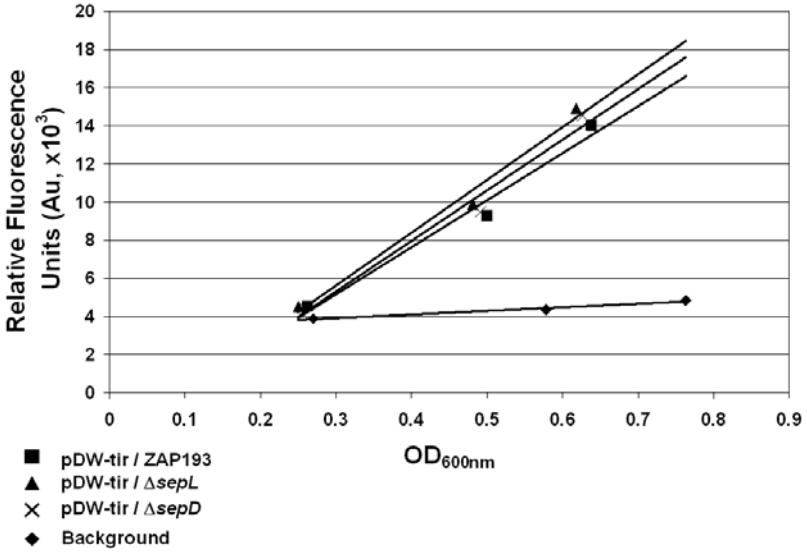


Fig. 2.5. Tir-GFP expression in different EHEC O157 genetic backgrounds. (A) Tir-GFP expression was determined in strains transformed with the *tir-gfp* fusion (pDW-tir) cultured in MEM-Hepes. Fluorescence expression was carried out as described in Materials and Methods. (■) Wild type EHEC O157 ZAP193 (pDW-tir); (▲) Δ*sepL* ZAP1211 (pDW-tir); (×) Δ*sepD* ZAP1144 (pDW6); (◆) EHEC O157 ZAP 193 (control, no *gfp* plasmid). Each expression experiment was carried out on at least three occasions and one replicate is shown. Data cannot be combined from different experiments as the sampling optical density points are different each time

2.2.5 SepD activates *sepL*/LEE4 transcription

It was observed that SepL expression was decreased in a *sepD* mutant strain. It raises the question as to whether SepD controls SepL expression at the transcriptional or post- transcriptional level. To answer this, transcription of *sepL* was investigated using a *gfp* fusion (pAJR74, Roe *et al.*, 2003b). This transcriptional fusion was transformed into different backgrounds and cultured in MEM-Hepes. The transcription of *sepL* (pAJR74) was reduced to the same extent as the full length SepL-GFP translational fusion (pDW6) (Fig. 2.6), indicating that SepD controls *sepL* expression at the transcriptional level.

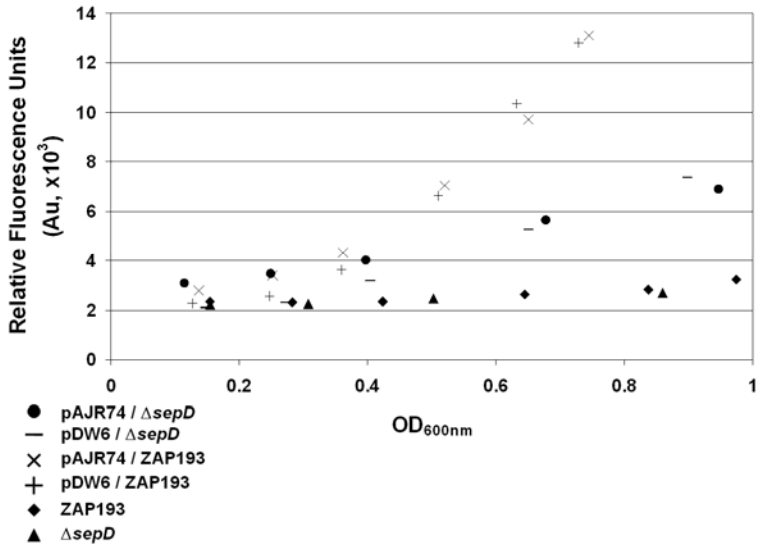


Fig. 2.6. SepD activates *sepL*/LEE4 transcription. (A) SepL-GFP expression was determined in strains transformed with the *sepL-gfp* fusion (pDW6) cultured in MEM-Hepes. Fluorescence expression was carried out as described in Materials and Methods. (●) (pAJR74); (—) Δ*sepD* ZAP1144 (pDW6); (┼) Wild type EHEC O157 ZAP193 (pDW6); (×) Wild type EHEC O157 ZAP193 (pAJR74); (◆) EHEC O157 ZAP 193 (control, no *gfp* plasmid); (▲) Δ*sepD* ZAP1144 (control, no *gfp* plasmid). Each expression experiment was carried out on at least three occasions and one replicate is shown. Data cannot be combined from different experiments as the sampling optical density points are different each time

2.2.6 *sepL* cleavage mutation doesn't change *sepL* expression level

Previous work in our laboratory had indicated post-transcriptional regulation of the LEE4 transcript, including cleavage of the transcript at the 3' end of *sepL* to release a distinct *espADB* transcript. This 'cleavage point' was previously mapped using primer extension analysis (Beltrametti *et al.*, 1999) and our laboratory (Dr. A. Roe, personal communication). Published research interpreted this cleavage site as a transcriptional start site for *espADB*, but subsequent research has shown that there is no promoter activity detectable adjacent to the *espADB* transcript and therefore this transcript is most likely to be generated by cleavage of a larger LEE4 transcript. Published research during my research has shown this to be the

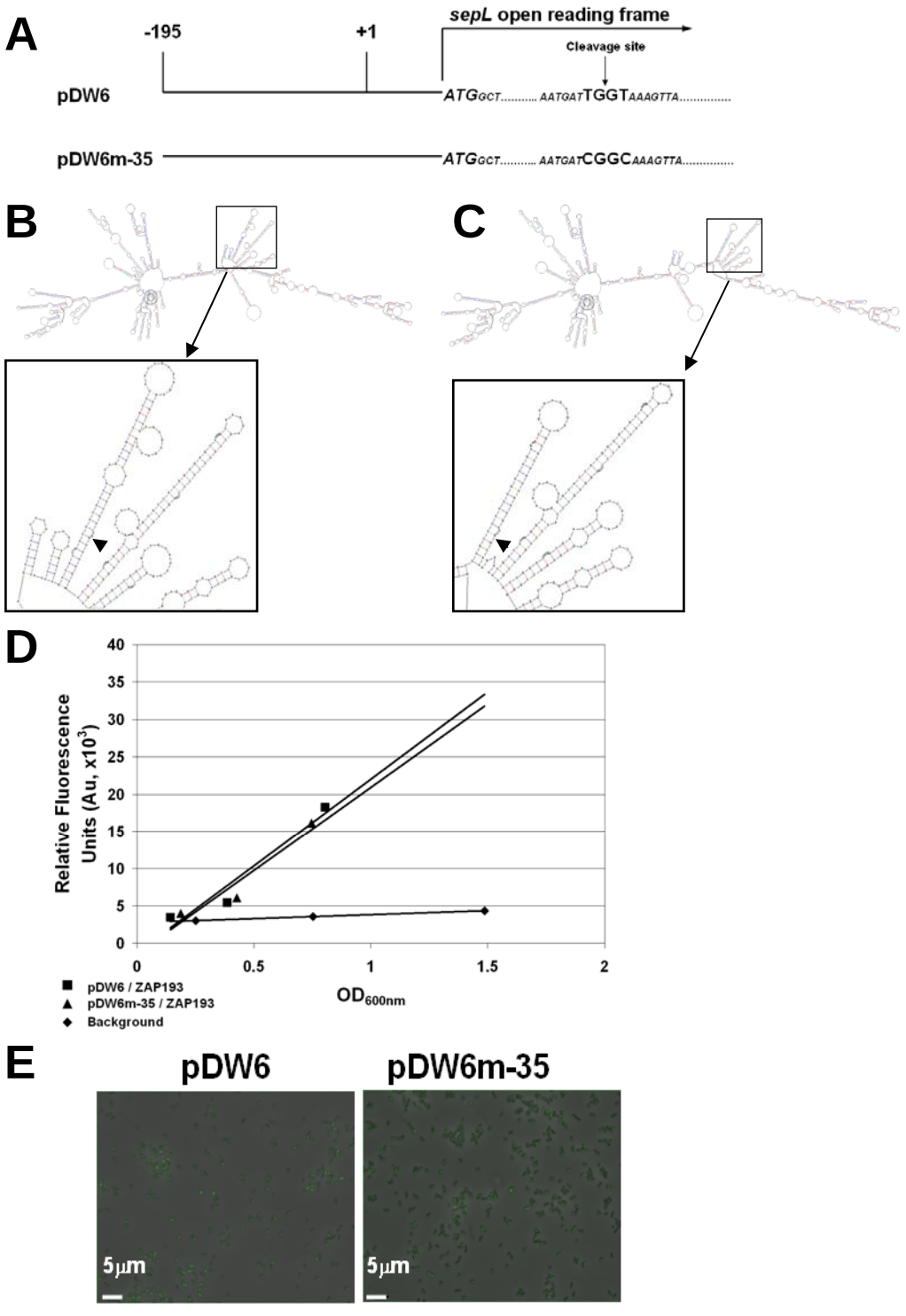


Fig. 2.7. Cleavage site mutation doesn't alter SepL-GFP expression
(A) Full length *sepL* and *sepL* cleavage site mutant all containing the *sepL* promoter region were translationally-fused to *gfp* and transformed into the high secretor strain *E. coli* O157 ZAP193. (B-C) Modelling of RNA folding was carried out for the different *sepL-gfp* RNAs using RNADraw. After introducing a synonymous mutation at the cleavage site within sepL ORF, the secondary structure was changed around the end of *sepL* transcript. (D) The total fluorescence values were examined for the effect of this synonymous mutation as described in Materials and Methods. Data cannot be combined from different experiments as the sampling optical density points are different each time. There are no differences in SepL-GFP expression between the original construct and the mutated construct (E). This result suggests that this synonymous mutation doesn't affect SepL expression.

case with the cleavage dependent on RNaseE activity (Lodato and Kaper, 2009). This processing might be vital to LEE4 post transcriptional control. There are numerous reports which indicate that mRNA structure is crucial for transcript cleavage. To examine this a synonymous mutation was introduced on pDW6 (full length SepL-GFP) to generate pDW6m-35 by site-directed mutagenesis around this cleavage site within *sepL* (Fig. 2.7A). Although pDW6m-35 still encodes the same SepL-GFP product, its mRNA structure around the cleavage site was changed to form a different predicted structure compared with the wild type fusion (Fig. 2.7B-C). However, it remains unknown whether this mutation would affect *sepL* mRNA processing. This altered full length SepL-GFP construct was transferred into EHEC ZAP193 and expressed under T3S permissive conditions. Total fluorescence was then measured as described in Materials and Methods. Compared with pDW6, the population expression level of pDW6m-35 remained unchanged under the conditions tested (Fig. 2.7D). As the construct did not show any difference in expression and as a result of time limitations, post-transcriptional processing of this mutated *sepL-gfp* mRNA was not examined by northern analysis. When checked by fluorescence microscopy, this mutant demonstrated a similar phenotype to pDW6 and no effect was observed on heterogeneous SepL-GFP expression (Fig2.7E).

2.2.7 Mutagenesis of the first base of the *sepL* transcript doesn't affect SepL expression

In order to study *sepL* sequence variation between different EHEC strains, previous work in our laboratory had sequenced a variant that gave elevated expression levels (>100 fold) of a SepL-LacZ fusion. This contained 3 mutations in the sequence preceding *sepL* when compared with *sepL* from

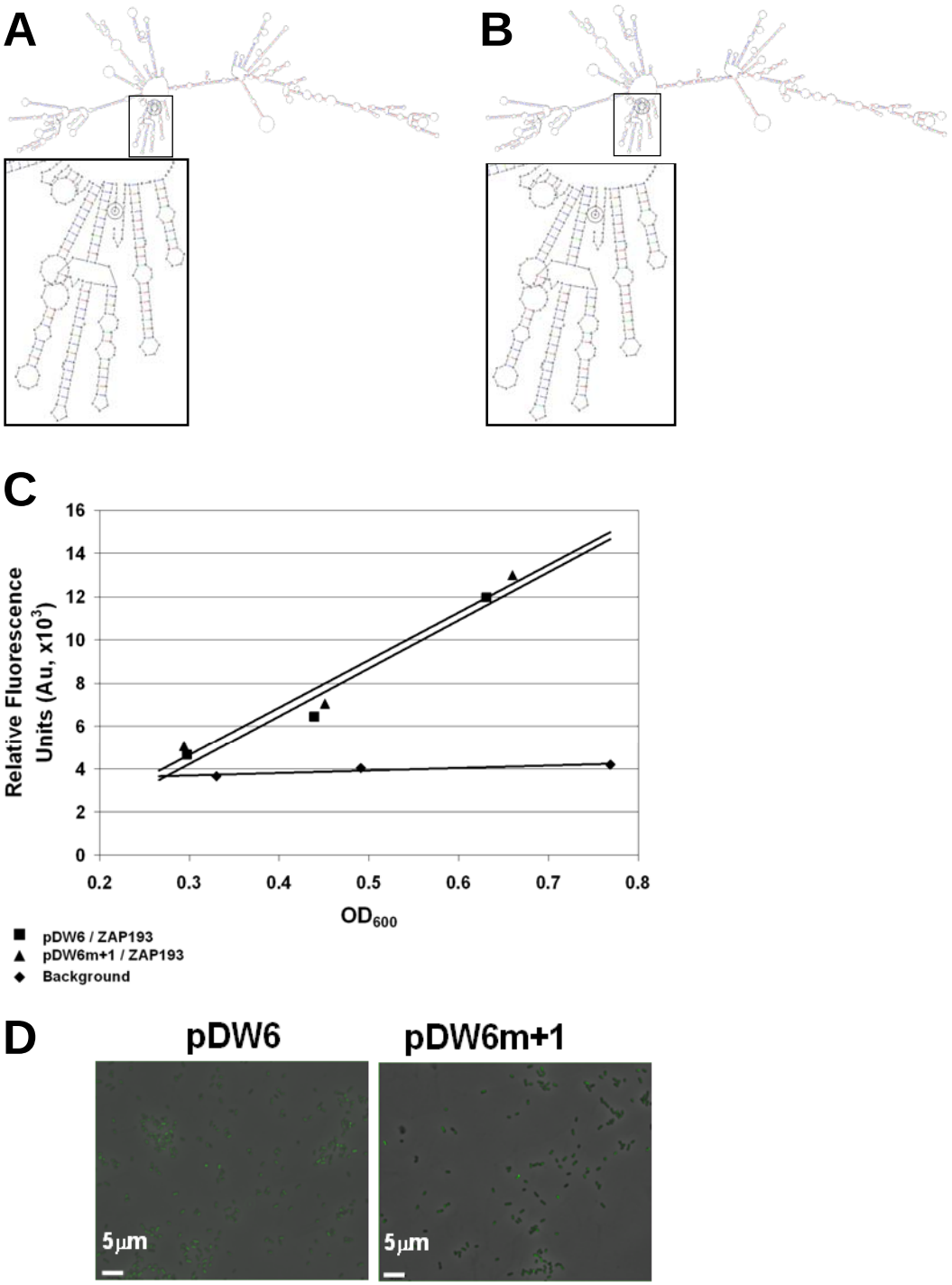


Fig. 2.8. +1 site mutation of *sepL* transcription doesn't alter SepL-GFP expression
Modelling of RNA folding was carried out for *sepL-gfp* wild type (A) and +1 mutant (B) RNAs using RNADraw. After introducing a T⟶C mutation at the +1 site of sepL transcript, the *m*RNA secondary structure remains unchanged (B). The total fluorescence values were examined for the effect of this synonymous mutation as described in Materials And Methods (C). Data cannot be combined from different experiments as the sampling optical density points are different each time. There are no difference in SepL-GFP expression between the original construct and the mutated construct (C-D). This result suggests that this mutation doesn't change SepL expression regulation.

EDL933. Two altered nucleotides (A→T at -69 and -115) were located upstream of the *sepL* transcript and one at the first base of *sepL* transcript. It has been shown that the +1 site is important in binding the RNA polymerase during transcription initiation. So, the T→C change at the +1 site of sepL *m*RNA could have a significant impact on SepL expression. To further characterise this mutant, the translational fusion plasmid (pDW6m+1) was constructed in which a C was introduced to replace T at the +1 site of the *sepL* transcript using site-directed mutagenesis (Fig. 2.8A-B). pDW6m+1 was then transformed into EHEC ZAP193 and SepL-GFP expression analysed. However, this +1 mutation also did not show any effect on SepL-GFP expression (Fig. 2.8C-D). This result suggests that the two changes adjacent to the *sepL* transcript could be having an important impact on *sepL* promoter activity or regulation. This remains to be investigated.

2.2.8 Mapping the area sufficient for SepL heterogeneous expression

To map the sequence which is crucial for the heterogeneous expression of *sepL*, full length *sepL*, the first 573, 210 and 51 base pairs of *sepL* were amplified with their own promoter and fused to *gfp* in frame (Fig. 2.9A). These constructs can therefore be used to examine the heterogeneous expression of *sepL* truncates. It was shown above that SepL was expressed at a very low proportion in EDL933. In order to double check the heterogeneous expression of *sepL* in another low secretor strain, the full length fusion was expressed in ZAP108, previously demonstrated to express a low proportion of EspA filaments under a T3 permissive condition (Roe *et al.*, 2003b). In this strain, only a low proportion of bacteria (<1%) were observed by fluorescence microscopy that expressed SepL-GFP (Fig. 2.9B).

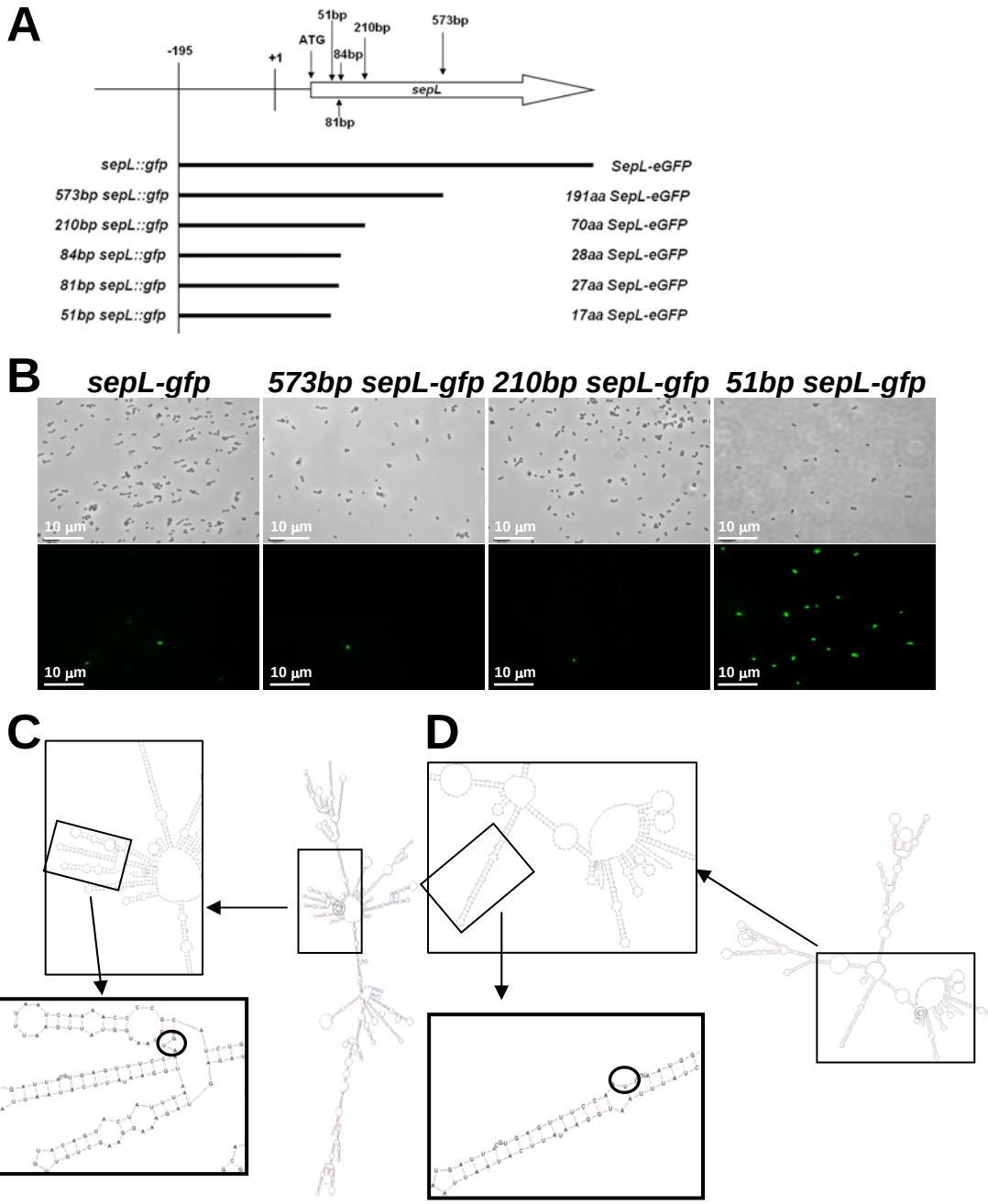


Fig. 2.9. Mapping the region of *sepL* required for post-transcriptional control
(A) Full length *sepL* and *sepL* carboxy terminal truncates all containing the *sepL* promoter region were translationally-fused to *gfp* and (B) transformed into the low secretor strain *E. coli* O157 ZAP108 (Table 1). Fluorescence in the bacterial population was imaged; heterogeneous expression was evident for the constructs containing full length sepL and the 573 bp and 210 bp truncates, with fluorescence detectable in only a small percentage of cells (<1%). By contrast, fluorescence was detectable in every bacterium containing 1 the 51 bp *sepL-gfp* fusion. (C-D) Modelling of RNA folding was carried out for the different *sepL-gfp* RNAs using RNADraw. The AUG start codon for *sepL* is sequestered between two hairpin loops for the full length, 573 and 210 bp constructs as indicated in the full length model shown in (C) with insets to indicate the AUG start codon that is circled. (D) The start codon is then exposed in constructs with less *sepL* coding sequence, such as the 51 bp construct indicated.

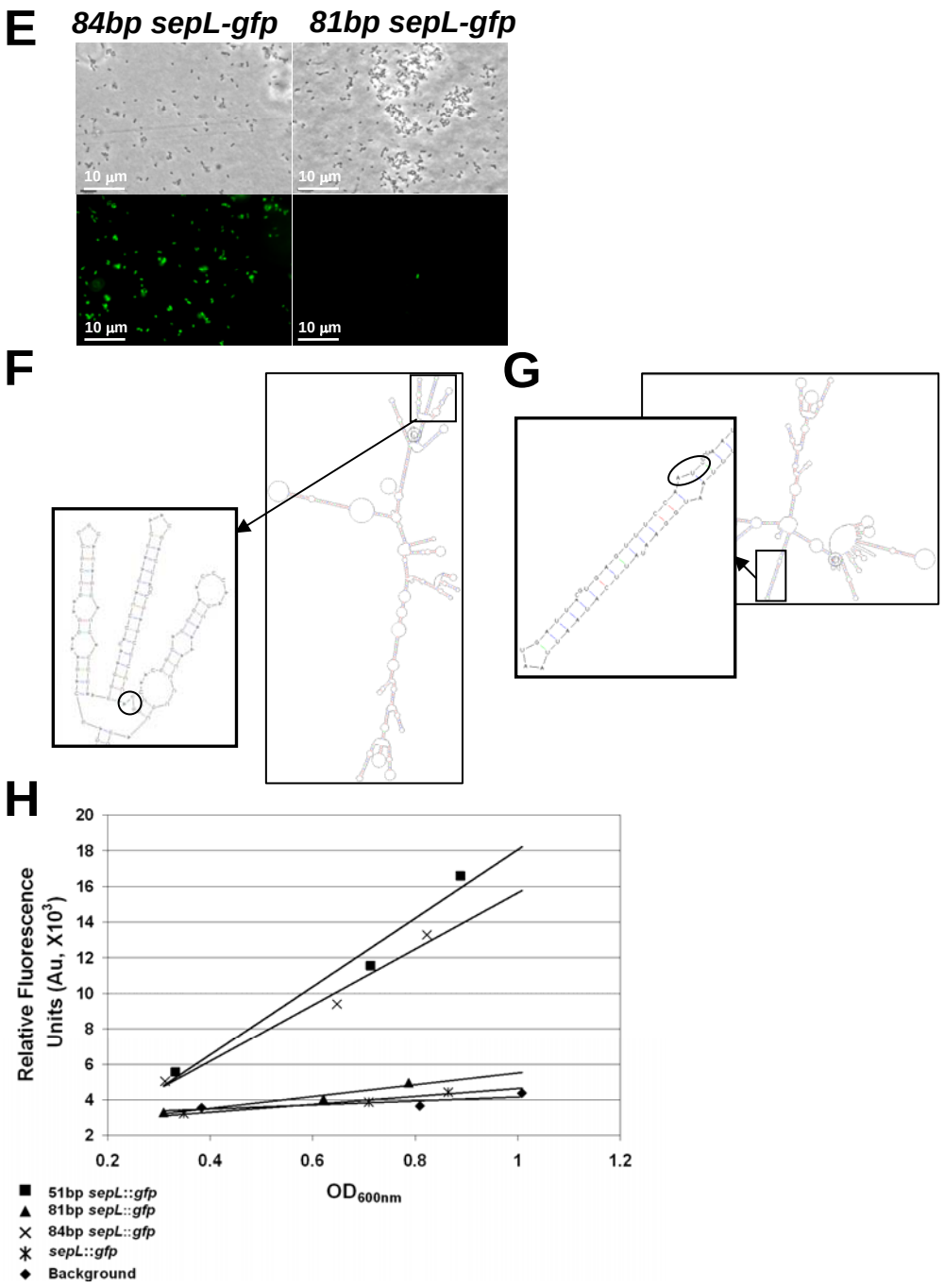


Fig. 2.9. Mapping the region of *sepL* required for post-transcriptional control
(E) Based on the RNA folding predictions, the structure transition indicated in (C-D) occurs when 81 bp of *sepL* are included in the modeling (G). To test this and define the minimum region of *sepL* required for heterogeneous regulation, the *sepL* promoter region and either 81 bp (E.G) or 84 bp (E,F) of the *sepL* open reading frame were fused to *gfp* and fluorescence examined in the low secretor strain ZAP108. As supported by population fluorescence measurements (H), the construct containing 81 bp of *sepL* was sufficient to restrict translation to a subset of the population as predicted (E). Of note is that the 84 bp *sepL* fusion was not restricted, with expression in every cell and increased levels at the population level (E-F). The marked difference between the translation of the 81 and 84 bp fusions indicates that this is the approximate transition point for the RNA structure to switch between the restrictive and non restrictive. Data cannot be combined from different experiments as the sampling optical density points are different each time.

Our data also showed that all SepL-GFP truncates which had 70aa or more of SepL could only be detected in a subset of bacterial cells (<1%) (Fig. 2.9B). A construct with the first 17aa of SepL (*51bpsepL-gfp*) fused to GFP was, by contrast, expressed in almost every individual bacterium (>99%) (Fig. 2.9B). These results suggest that the first 51 base pairs of *sepL* do not contain sufficient information to result in heterogeneous expression of SepL by comparison with the other truncates.

As previous research has revealed that the structure of target mRNA is very important for a small RNA regulation of transcripts and since the expression of the different length *sepL* constructs varied, I used RNA modelling software (RNAdraw v1.1) to predict the mRNA structure of these truncates (all fused to gfp). These models suggest that there is a significant change around the *sepL* AUG intiation codon when the 51 base pair construct is compared with the longer *sepL* sequences (Fig. 2.9C, D). Therefore combining these two observations, it indicates that a change in *sepL* mRNA structure may be playing an important role in controlling the heterogeneous expression of SepL.

In order to define the minimum sequence required for this post-transcriptional control, further RNA structural modelling of different length *sepL* fusions was carried out. According to this, the mRNA structure reverts back to the full length folding pattern once 84bp of *sepL* are included in the model (Fig.2.9F-G). As a consequence, I made 81bp and 84bp *sepL-gfp* constructs to test this prediction. It was demonstrated under the fluorescence microscope that 84bp *sepL-gfp* construct was expressed in every bacterium

as with the 51bp *sepL-gfp* construct (Fig. 2.9E). However, the 81bp *sepL-gfp* construct was only expressed in a subset of cells as with the full length *sepL-gfp* construct (Fig. 2.9E). Population-based fluorescence readings also supported different translational patterns in these two constructs (Fig. 2.9H). It is clear as shown in Fig. 2.9E-H that such a slight change has a dramatic impact on expression/regulation.

2.2.9 Analysis of short SepL-GFP fusions

In addition to the truncates described above, different length *sepL* leading sequences were fused to *gfp* spanning from the first codon to 48bp of *sepL*. These 16 short *sepL-gfp* fusions were then transformed into EHEC strain ZAP193 and cultured in MEM-Hepes. Samples were taken at different time point, and the OD_{600nm} / total fluorescence readings were measured as described in Materials and Methods. When the OD_{600} of these cultures reached 0.6, the sample was fixed with 4% PFA and fixed to slides. As shown before, GFP production was observed in every bacterium when the first 51bp *sepL* was fused to *gfp*. Unexpectedly, it was found out that the shortest *sepL*::*gfp* fusion (pDWsepL3) only showed background GFP production although the other 15 fusions (pDWsepL6, pDWsepL9, pDWsepL12, pDWsepL15, pDWsepL18, pDWsepL21, pDWsepL24, pDWsepL27, pDWsepL30, pDWsepL33, pDWsepL36, pDWsepL39, pDWsepL42, pDWsepL45 and pDWsepL48) can give a high GFP production in EHEC (Fig. 2.10B). The same result was also evident when the different constructs were examined by fluorescence microscopy. Fluorescence was detected in the majority of bacterial cells for all the 16 fusions except the shortest. pDWsepL3 and pDWsepL6 both have the *sepL*

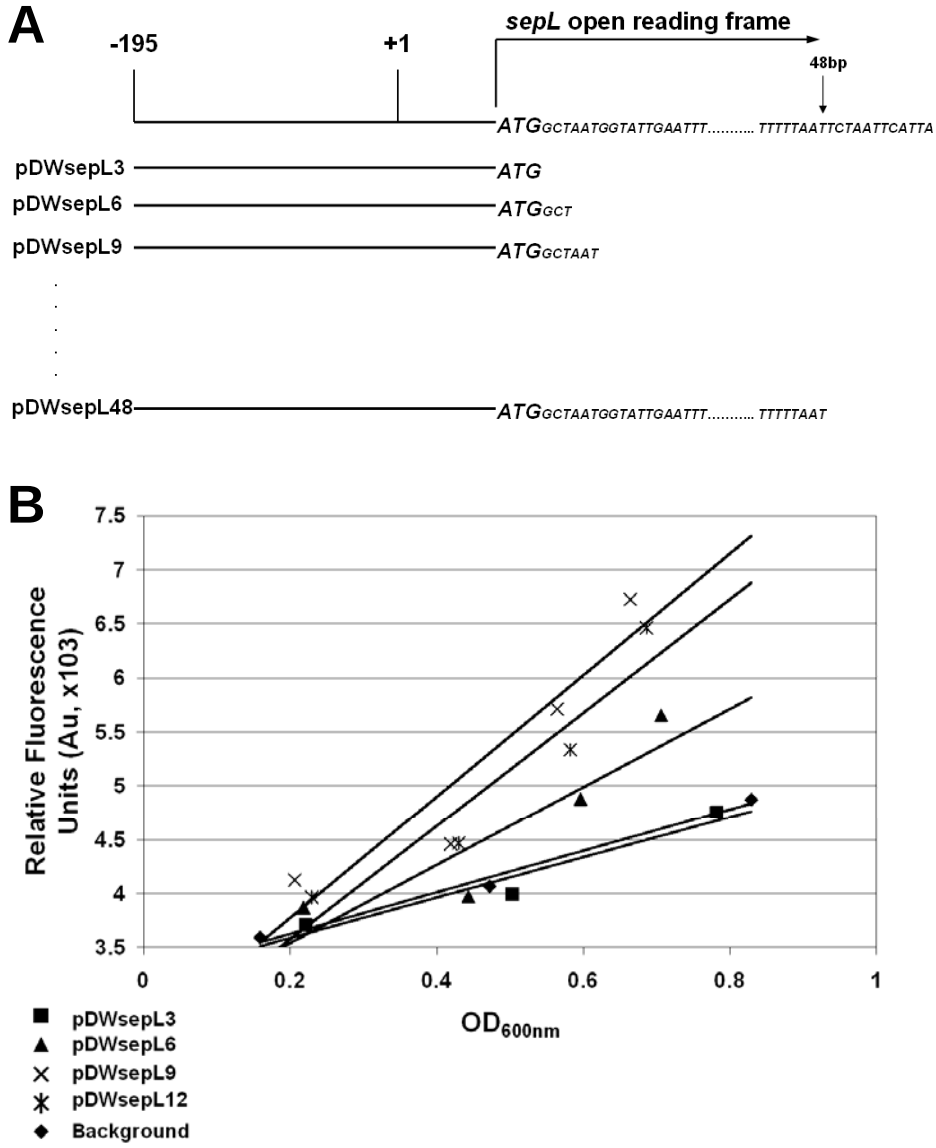


Fig. 2.10. Analysis of short SepL-GFP fusions (A) *sepL* carboxy terminal truncates all containing the *sepL* promoter region were translationally-fused to *gfp* and (B) transformed into the high secretor strain *E. coli* O157 ZAP193 (Table 1). Unlike the shortest *sepL* (pDWsepL3) (■) fusions, other *sepL* fusions (pDWsepL6, pDWsepL9, pDWsepL12) were expressed at high levels (▲, ×, ✳) compared to ZAP193 without the fusion construct (◆).Data cannot be combined from different experiments as the sampling optical density points are different each time.

native promoter and 5' UTR (Fig. 1.9 and 2.10A). pDWsepL3 only contains the first 3 base pairs (ATG start codon) of the *sepL* gene but pDWsepL6 contains the first 6 base pairs. Although there is only a difference of 3 base pairs between pDWsepL3 and pDWsepL6, when expressed in EHEC O157,

they provided two totally different expression patterns. This result suggests that the area around *ATG* start codon plays a vital role in *sepL* expression regulation.

2.2.10 Replacement of the *sepL* 5'UTR impaired SepL expression

As demonstrated above, although the expression of full length SepL-GFP can only be observed in certain cells, a short *sepL* region (the first 51 base pair of *sepL* ORF) including its 298 base pair promoter sequence when fused to the *gfp* reporter gene was expressed in every cell as determined by fluorescence microscopy (Fig. 2.9B). It indicates that this short *sepL* fusion no longer contains the required sequence for SepL heterogeneous expression. Therefore, the leading mRNA sequence of *sepL* is not being post-transcriptionally regulated in the same way as the full length *sepL* mRNA sequence. Post-transcriptional regulation of virulence determinants is common and may involve regulatory factors binding to the mRNA sequence. Although the extra factors can mediate the regulation by binding to the 5' sequence of the gene coding area, they usually bind upstream of the gene coding sequence, the 5' untranslated region (UTR) in most cases. For this work, I tested whether the specific 5' UTR used is crucial for SepL expression.

A hybrid construct was made to investigate the importance of this region. The full length *sepL* coding sequence including the *ATG* start codon was amplified from EHEC O157 strain ZAP193 and cloned into pAJR70 to generate an in frame fusion to *gfp* (pDW6m). A *sepL* upstream sequence including its native promoter region was then amplified from EHEC O157

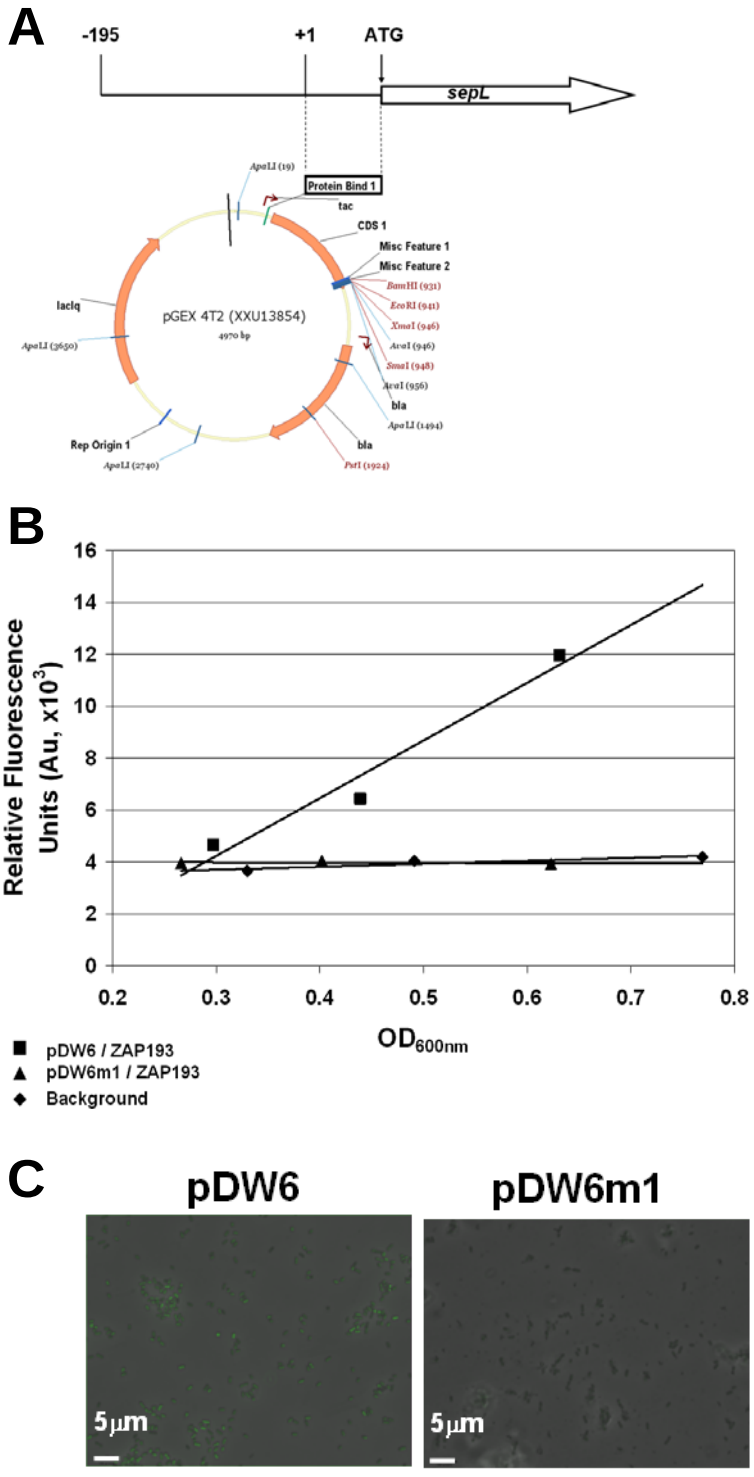


Fig. 2.11. Replacement of *sepL* 5'UTR impaired SepL expression (A) A full length *sepL-gfp* plasmid in which *sepL* 5'UTR was replaced by an artificial sequence from expression vector pGEX4T2 was transformed into the high secretor strain *E. coli* O157 ZAP193 genetic background (◆) . (B-C) Unlike another full length *sepL-gfp* fusion (pDW6) (■), this fusion was expressed at extremely low level (▲).Data cannot be combined from different experiments as the sampling optical density points are different each time.

strain ZAP193 and inserted in front of *sepL* in pDW6m to generate pDW6m0. As the final step, an artificial 5' UTR including a ribosome binding site was then amplified from a well known expression vector ─ pGEX4T2 and restricted into pDW6m0 between the *sepL* promoter and coding sequence to generate pDW6m1. In summary, the final construct (pDW6m1) keeps almost all the DNA features of the full length *sepL-gfp* construct (pDW6) except it contains a replaced 5' UTR region (Fig. 2.11A). pGEX 4T2 is a commercial vector normally used to express GST-fused proteins. It has an IPTG-inducible promoter and an optimised ribosome binding site for GST-fusion protein expression. In this study, the sequence containing this optimised ribosome binding site (RBS) for GST replaces the original *sepL* 5' RBS. This hybrid construct-pDW6m1 was put into the same host strain-ZAP193, which was used for studying *sepL* expression in the previous experiments and cultured in MEM-Hepes (T3S pemissive condition). At different time points, samples were taken from the culture to measure the optical density at 600nm and then total fluorescence of each sample was measured. Fluorescence measurement was carried out as described in the Materials and Methods section. By comparison with pDW6, this chimeric fusion produced extremely low fluorescence levels. At OD_{600}=0.8, the GFP expression level of pDW6m1 was reduced by a 100 fold compared with pDW6 (Fig. 2.11B-C). This interesting observation is more likely considered as a logical consequence of the sequence difference between those two constructs. Therefore, the replacement of *sepL* upstream sequence, primarily the RBS, more or less negates expression of this fusion.

2.2.11 LEE4 transcript under a post transcriptional regulation

It has been reported that different sized LEE4 transcripts can be detected using a LEE4 (*sepL*) probe (Roe *et al.*, 2003a, Beltrametti *et al.*, 1999). As shown above, the leading sequence of *sepL* might be important for SepL production. Therefore, a *sepL* partial deletion strain ─ M10 was made to test this hypothesis using allelic exchange. The first 10 base pairs of *sepL* ORF were replaced by a 6 bp *BamH* I restriction site. This deletion results in a reading frame change of *sepL* but otherwise leaves the rest of the LEE4 DNA sequence unchanged. The sequencing result showed that there were three unexpected changes (Listed in Table 5.2) within the C-terminus of *espA* gene in this construct. However, those changes are unlikely to change the manner of LEE4 mRNA regulation as LEE4 expression was not changed in a Δ*espA* strain (Deng *et al.*, 2004). Unlike other *sepL* mutants, M10 shows a polar effect and cannot be complemented by *sepL* expressed from a plasmid. In this study, total mRNA was extracted from the different strains cultured in MEM-Hepes and equal amounts of total mRNA loaded onto a gel. A Northern analysis was carried out using a *sepL* probe as described in Materials and Methods. This Northern blotting of the EHEC wild type strain gave a result consistent with previous reports. There are two major bands detected using a *sepL* probe. The large transcript is about 4 Kb and the small transcript is about 1.1 kb (Fig. 2.12, panel 1). For a *sepD* mutant in which the whole *sepD* ORF was removed from EHEC chromosome, these two bands were detected and the ratio of two transcripts remained similar. The only obvious change in the *sepD* mutant is that the overall density of detectable *sepL* transcripts is much weaker compared to the wild type. It also provides further evidence that *sepD* affects *sepL* expression at a

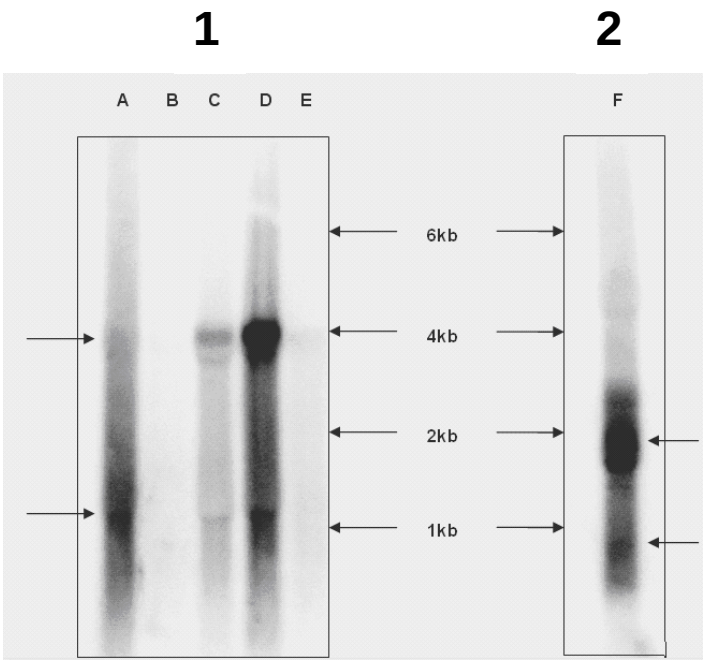


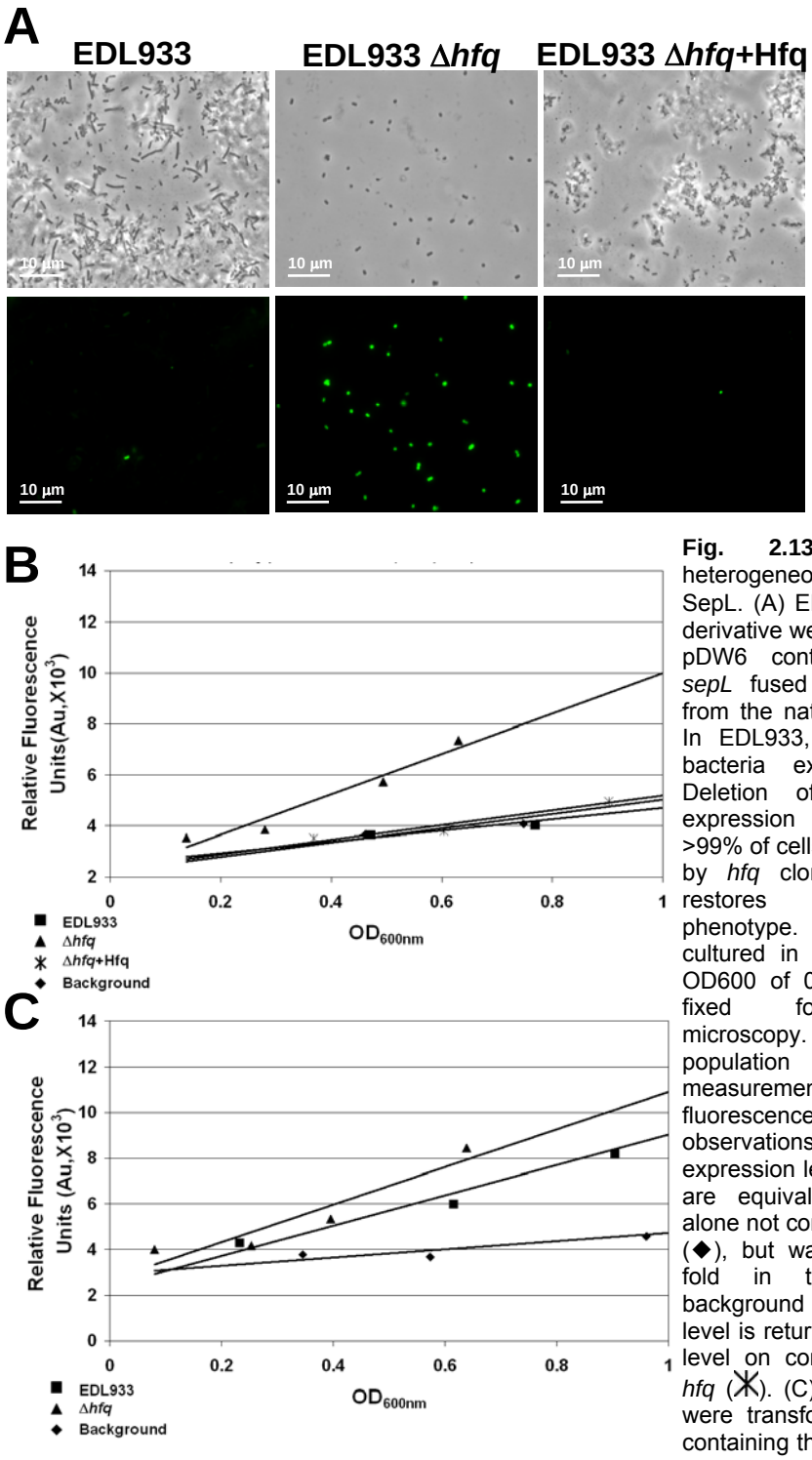
Fig. 2.12. Northern analyses of LEE4 operon expression. Detection of transcript mRNA with a *sepL* probe (1) in different strains and culture conditions: A. M10 (MEM-Hepes); B. Δ*LEE4*; C, Δ*sepD* (MEM-Hepes); D, ZAP193 (MEM-Hepes); E, ZAP193 (LB).
Panel 2 shows: pDW6 was transferred into WT EHEC (ZAP193, MEM-Hepes). And mRNA transcript was detected with *gfp* probe (F).

transcriptional level and not post-transcriptionally as indicated by analysis of the GFP fusions (Fig. 2.6). Moreover, the M10 RNA sample gave a totally different pattern when blotted with the same *sepL* probe. For M10, only the 1.1kb band was detected (Fig. 2.12, panel 1). This result suggested that the LEE4 transcript was regulated differently in the M10 mutant compared to the wild type strain. The SepL-GFP construct was also analysed by Northern blotting using total mRNA extracted from bacteria cultured in the same way as above and a *gfp* probe. This revealed 2 major bands (Fig. 2.12, panel 2). These two detected bands were in a ratio comparable to those shown for the wild type *sepL* transcript. This result suggests that *sepL-gfp* transcript is also cleaved the same way as the LEE4 transcript and therefore

the fusion is a good indicator of WT *sepL* transcript expression and processing.

2.2.12 Hfq regulation of SepL translation

From the results above, it is evident that *sepL* mRNA folding and processing are critical for SepL heterogeneous expression. One strong possibility is the involvement of a small regulatory RNA. Recent published work suggests that RNaseE is required for LEE4 transcript processing. Hfq, a small RNA chaperone is a global regulator of gene expression, and can be involved in RNaseE-related mRNA degradation. It is known that EDL933 *hfq* mutant has an enhanced pedestal formation phenotype, equivalent to an EDL933 *dam* mutant (Prof. Martin Marinus, personal communication). Given this information, it was interesting to test SepL expression in an *hfq* mutant. Therefore, the full length SepL-GFP fusion was transformed into the wild type, Δ*hfq* and *hfq* complemented strains which were then imaged by fluorescence microscopy (Fig. 2.13A). In the *hfq* deletion background, expression of the SepL-GFP fusion is clear in the majority of bacteria (>99%) compared to only approx. 1% in the wild type EDL933 strain. The proportion of bacteria expressing the fusion in Δ*hfq* was then reduced following complementation with Hfq. This result is in agreement with population level measurements of SepL, including Western blotting (unpublished data, Xu and Gally) and culture fluorescence levels (Fig. 2.13B) which were elevated 100 fold in an *hfq* mutant. By contrast, expression from the 51 bp *sepL-gfp* fusion was increased modestly (less than 1.5 fold) demonstrating the importance of the extended *sepL* region for *hfq* regulation (Fig. 2.13C).



Data in B and C cannot be combined from different experiments as the sampling optical density points are different each time

Fig. 2.13 Hfq controls heterogeneous expression of SepL. (A) EDL933 and its Δ*hfq* derivative were transformed with pDW6 containing full length *sepL* fused to *gfp* expressed from the native *sepL* promoter. In EDL933, less than 1% of bacteria express SepL-GFP. Deletion of *hfq* results in expression of SepL-GFP in >99% of cells. Complementation by *hfq* cloned into pWSK29 restores the wild-type phenotype. Bacteria were cultured in MEM-Hepes to an OD600 of 0.8 and the culture fixed for fluorescence microscopy. (B) Whole cell population fluorescence measurements confirm the fluorescence microscopy observations. SepL-GFP expression levels in EDL933 (■) are equivalent to the strain alone not containing the plasmid (◆), but was raised over 100 fold in the *hfq* deletion background (▲). This elevated level is returned to the wild type level on complementation with *hfq* (✳). (C) EDL933 and Δ*hfq* were transformed with pDW26 containing the first 51bp of *sepL* fused to *gfp* expressed from the *sepL* promoter. Unlike the full length *sepL* fusion, this construct was expressed at high levels (■) compared to EDL933 without the fusion construct (◆) and this level of expression was increased only slightly (<1.5 fold) in the absence of *hfq* (▲).

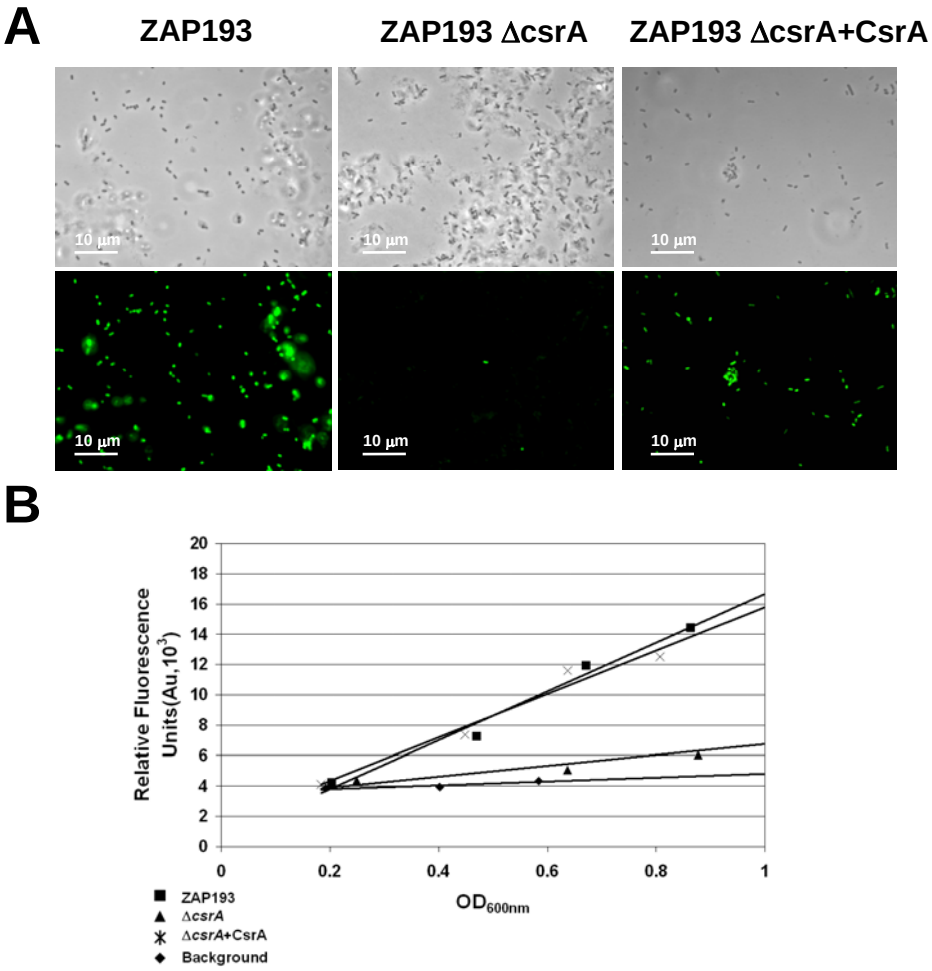


Fig. 2.14. CsrA controls heterogeneous expression of SepL. (A) ZAP193 and its Δ*csrA* derivative were transformed with pDW6 containing full length *sepL* fused to *gfp* expressed from the native *sepL* promoter. In ZAP193, >80% of bacteria express SepL-GFP. Deletion of *csrA* results in expression of SepL-GFP in less than 1%of cells. Complementation by *csrA* cloned into pWSK29 restores the wild-type phenotype. Bacteria were cultured in MEM-Hepes to an OD600 of 0.8 and the culture fixed for fluorescence microscopy. (B) Whole cell population fluorescence measurements confirm the fluorescence microscopy observations. SepL-GFP expression levels in ZAP193 (■) are equivalent to the strain alone not containing the plasmid (◆), but was decreased by 10 fold in the *csrA* deletion background (▲). This elevated level is returned to the wild type level on complementation with *csrA* (✳).Data cannot be combined from different experiments as the sampling optical density points are different each time.

2.2.13 CsrA regulation of SepL translation

CsrA is known as a post-transcriptional regulator which mediates the binding between sRNA *csrBCD* and its chaperone protein, Hfq. It has been revealed that CsrA plays a key role in the adaptation of bacterial pathogens to different stages of infection in animals (Lucchetti-Miganeh *et al.*, 2008).

An EHEC O157 *csrA* mutant was generated by allelic exchange and tested for heterogeneous SepL expression. The expression of SepL was decreased dramatically in this mutant at the population level. Comparing expression with the parental EHEC ZAP193 wild type strain, the expression of SepL-GFP is reduced more than 20 fold in a *csrA* deletion mutant (Fig. 2.14B). When the heterogeneity of SepL expression was examined by fluorescence microscopy, it was evident that less than 1% of Δ*csrA* bacterial cells expressed SepL-GFP (Fig. 2.14A). While for the high secretor parent strain (ZAP193), SepL-GFP expression was observed in more than 80% of cells (Fig. 2.14A). This data demonstrates that CsrA, directly or indirectly, is involved in the regulation of SepL expression.

2.3 Discussion

As suggested by previous work in our laboratory, T3S is important for the colonization of EHEC at the terminal rectum of cattle and the expression levels of T3S proteins EspD, EspA and Tir are variable between different EHEC strains (McNally *et al.*, 2001, Roe *et al.*, 2003b, Naylor *et al.*, 2003, Naylor *et al.*, 2005). This difference is exemplified by imaging the secretion of T3 translocon proteins, especially EspA filaments, on the bacterial surface. This variability has been suggested to potentially affect shedding levels from cattle and therefore the likelihood of subsequent human infection (Chase-Topping *et al.*, 2008). So far, it has been shown that T3 secretion is regulated by different multiple inputs which can act at transcriptional, post-transcriptional and post-translational levels. This variation between strains is likely to be a total sum of various inputs from these three levels.

Different factors were reported to activate or repress T3SS of EHEC/EPEC. Some factors, such as Ler and H-NS, regulate T3S by directly activating or repressing LEE transcription. H-NS has been shown to repress LEE expression of EPEC (Umanski *et al.*, 2002). Therefore, as an H-NS homologue, Ler is acting as an anti H-NS factor to activate LEE expression (Bustamante *et al.*, 2001). Moreover, Ler has been shown to be important for switching on expression of the non-LEE encoded T3 effector NleA (Elliott *et al.*, 2000). On the other hand, many factors, such as GrlA, Hha and RpoS, were found to regulate T3S indirectly via Ler (Juarez *et al.*, 2000, Sharma and Zuerner, 2004, Barba *et al.*, 2005, Laaberki *et al.*, 2006, Dong and Schellhorn, 2009b). In the past, Ler has been shown to have an impact on controlling expression from LEE1, 2, 3, 5 and also the *esp* genes (Sperandio *et al.*, 2000, Sanchez-SanMartin *et al.*, 2001, Haack *et al.*, 2003, Sharma and Zuerner, 2004). The effect of Ler on *sepL* expression has not been mentioned since *esp* genes were thought to be transcribed from their own promoters instead of processed from a larger LEE4 transcript (Beltrametti *et al.*, 1999). However, later work in our group and other groups suggested that two different size *espADB* transcripts result from post transcriptional cleavage of this transcript (Roe *et al.*, 2003b, Roe *et al.*, 2004, Lodato and Kaper, 2009). From my research, the expression level of SepL-GFP was reduced more than 10 fold in a *ler* deletion background and this implies that Ler has a major impact on LEE4/SepL expression. SepD deletion also results in a reduction of *sepL* transcription in EHEC. It has been demonstrated that the LEE4 operon is controlled post-transcriptionally and that the proportion of bacteria within a population that are engaged in T3S correlates well with high and low secreting phenotypes (Roe *et al.*, 2003b, Roe *et al.*, 2004, Lodato and Kaper, 2009). It is likely that the T3S phenotype is regulated not

only transcriptionally but also post transcriptionally in different EHEC isolates.

PerC, which is encoded by the *perABC* operon on the pEAF plasmid of EPEC, is a positive regulator of EPEC T3SS which modulates LEE expression through the LEE1/*ler* promoter and *perABC* operon was only found in EPEC (Gomez-Duarte and Kaper, 1995). *PchA,B,C*, three PerC-homologs which are encoded within cryptic prophages, were shown to act as positive regulators of LEE1 in EHEC (Porter *et al.*, 2005). Recent work has shown that there are variable regions adjacent to certain *pch* loci and Pch expression can therefore differ between strains leading to altered levels of LEE1 expression (Yang *et al.*, 2009). Although LEE1 expression has a considerable impact on T3S expression, it is unlikely that Pch variation controls LEE4 heterogeneous (post transcriptional) expression since LEE4 heterogeneous expression was not changed in a *ler* mutant as investigated in this study. However, it remains a possibility that the Pch level could impact on LEE4 translation by altering expression of other regulatory factor(s).

In my study, it was confirmed that EspA filament expression is heterogeneous and this correlated with T3S levels in various EHEC strains as reported (Roe *et al.*, 2003b, Roe *et al.*, 2004). SepL is essential for translocon secretion and switching from translocon to effector protein export (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Deng *et al.*, 2005, Wang *et al.*, 2008). As the first gene on the LEE4 operon, SepL expression was only found in the subset of cells that had EspA filaments on the surface. Results of Northern analyses suggested that the *sepL* transcript was also being post-transcriptionally processed as shown above (Fig. 2.12).

My experiments further revealed that the first part of the *sepL* reading frame (51bp*sepL-gfp*, Fig. 2.9B) is modulated in a different way compared with full length *sepL* transcript. By modelling the RNA structure, it was evident that the sequences present the AUG initiation codon differently. I propose that the sequestration of the initiation codon in the full length transcript, is linked to the restricted heterogeneous expression and this does not occur with the more open access to the codon provided in the shorter construct. Further modelling proposed the transition for this change change would be around 81-84 bp of *sepL* sequence. In order to test this theory, two SepL-GFP fusions (81bp *sepL-gfp* and 84bp *sepL-gfp*) were made based on the RNA modelling and expressed under a T3S permissive condition. A clear difference was found in the expression pattern between 81bp and 84bp *sepL* constructs. The heterogeneity of SepL expression was not apparent with the 84bp *sepL-gfp* construct but was still heterogeneous with the 81bp *sepL-gfp* construct (Fig. 2.9E). The fact that 3 extra bases can modulate SepL expression pattern dramatically supports my hypothesis.

Hfq is a global regulator which is recruited by sRNA factors involved in post-transcriptional regulation (Nogueira and Springer, 2000, Sittka *et al.*, 2008, Sittka *et al.*, 2009). It was demonstrated that Hfq repressed EHEC T3S in my study and the heterogeneity of SepL expression was also changed in the absence of Hfq (Fig. 2.13). According to this data, I propose an Hfq-dependent regulation of T3S in EHEC which is also supported by two very recent publications (Shakhnovich *et al.*, 2009, Hansen and Kaper, 2009). The impact of Hfq on LEE1 (Ler regulation) was examined in detail in these two studies and it was found Ler was over-expressed in an *hfq* mutant. However,

it was not mentioned in their studies that Hfq is important for LEE4 heterogeneous expression. From my data, Hfq is also considered as a regulator of the LEE4 transcript independently of Ler. However, a co-factor might be required for Hfq to regulate LEE4 expression as Hfq were known associated to other factors (RNA or protein) (Vytvytska *et al.*, 1998, Sledjeski *et al.*, 2001, Moll *et al.*, 2003a, Sukhodolets and Garges, 2003, Folichon *et al.*, 2003, Moll *et al.*, 2003b). A posttranscriptional regulator, CsrA, was then examined for LEE4 heterogeneous expression regulation in my study. It was found that CsrA acts as an activator of EHEC T3S (Fig. 2.14). A similar observation is also reported by another group recently (Bhatt *et al.*, 2009) showing that CsrA binds to the 'leading sequence' of the *sepL* transcript. My results discovered that the 5' UTR and 5' *sepL* sequence also has a significant impact on SepL expression (Fig. 2.10 and 2.11) and it was interesting that the two proposed CsrA binding sites are included in this region (Bhatt *et al.*, 2009). Collecting all the information, it seems that CsrA binding could sequentially switch on LEE4 expression and Hfq would interfere with this binding directly or indirectly. Although it is still not fully understood how exactly LEE4 is post-transcriptionally regulated in EHEC, it is likely that Csr-sRNA regulators control LEE4 expression by direct binding requiring Hfq and a rough model is proposed in Fig. 2.15.

Hfq, which is normally associated with the bacterial RNA degradosome (Viegas *et al.*, 2007), might not be just acting as an sRNA chaperone protein in LEE4 expression repression. It is potentially involved in LEE4 transcript turnover as well. As suggested by previous research, two main factors of the RNA degradosome, PnPase and RNaseE were both critical for T3SS-

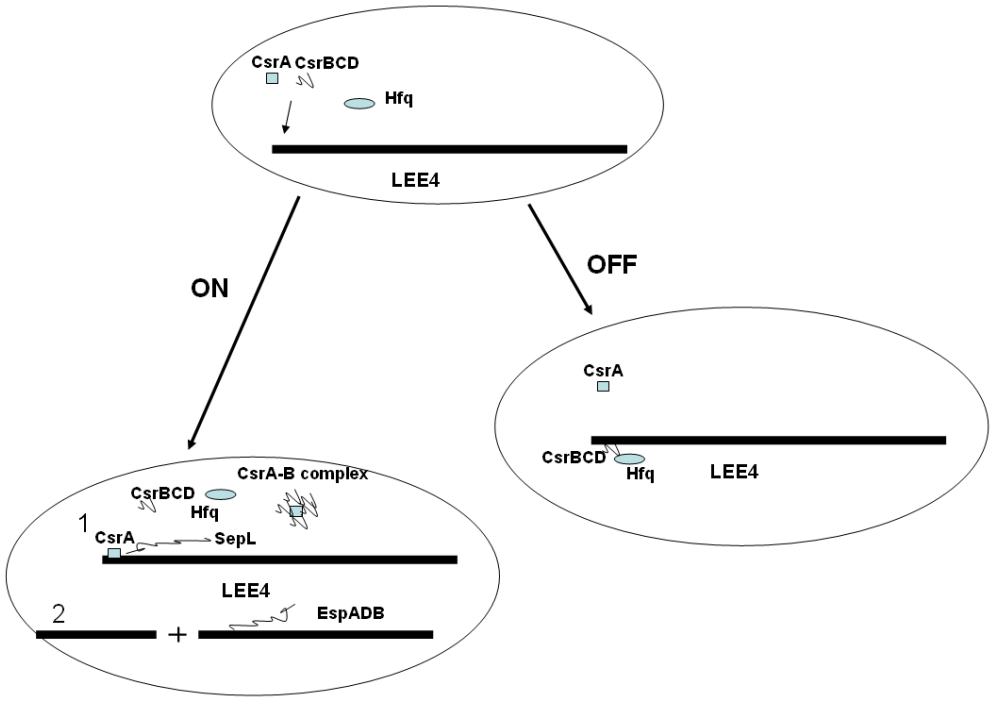


Fig.2.15. A simple model was proposed to elucidate the regulation of LEE4 heterogeneous expression. LEE4 translation is activated when CsrA binds to the LEE4 transcript and/or inhibits Hfq-mediated CsrB sequestration via CsrA/CsrB binding ('ON'). The large LEE4 (*sepL-espADB*) mRNA is cleaved by RNaseE after SepL is translated and then EspADB translation is initiated. LEE4 expression is repressed when CsrB binds to LEE4 transcript with its chaperone Hfq ('OFF'). The Large LEE4 mRNA is sequestered and no translation is initiated.

enhancing activity in *Yersinia* (Rosenzweig *et al.*, 2007, Rosenzweig and Schesser, 2007, Yang *et al.*, 2008) and it was proposed that the cleavage of LEE4 transcript is regulated by RNaseE as well (Lodato and Kaper, 2009). So RNA degradosome components probably coordinate LEE4 expression with sRNA regulators. Current ongoing work in the laboratory is mapping all nucleic acid sequences interacting with Hfq/CsrA to further understand LEE4 expression regulation.

Chapter 3

Studies to investigate the function of SepL:

including interactions between SepL/SepD and T3S apparatus and effector proteins

3.1 Introduction

T3SS is required for the formation of typical attaching and effacing lesions following EHEC, EPEC and CR infection of eukaryotic cells. This phenotype is mainly associated with bacterial attachment and pedestal formation on epithelial cells. It requires that the EspBD translocon proteins form a pore in the host cell membrane and open up an EspA conduit between the bacterium and host cell. After that, the translocated intimin receptor (Tir) is delivered into the enterocyte (Kenny *et al.*, 1997a, Deibel *et al.*, 1998) by the T3SS and Tir, together with other effector proteins, co-ordinates rearrangements of the cytoskeleton. The whole infection procedure includes 1. T3SS activation, i.e. expression of the T3SS; 2. EspA filament assembly/elongation that requires translocon export and assembly on the bacterial surface; 3. EspDB pore formation that opens up a T3SS conduit; 4. A switch from translocon protein to effector protein secretion, potentially following detection of a signal indicating that a conduit has been opened. The T3S system is evident as a needle-like projection on the surface of the bacterium (Tamano *et al.*, 2000, Sekiya *et al.*, 2001, Daniell *et al.*, 2001). Its proposed model has the basal apparatus proteins: EscD (Pas), R, S, T, U and V in the inner membrane and an outer membrane ring of EscC (Sekiya *et al.*, 2001, Ogino *et al.*, 2006). EscF may form a needle-like structure at the base of the EspA filament (Daniell *et al.*, 2001, Yip *et al.*, 2005b, Ogino *et al.*, 2006). The export of Tir and other effector proteins occurs through the hollow filament, with the timing and regulation of translocation potentially driven by chaperone proteins (Wainwright and Kaper, 1998, Elliott *et al.*, 1999a, Neves *et al.*, 2003a). Therefore, translocation of effector proteins into the host cell relies on the expression and assembly of EspADB. The LEE

pathogenicity island contains at least 41 genes in five main operons and all the T3SS structural proteins are encoded by genes on the LEE. The *espADB* genes are included within the LEE4 operon adjacent to the first gene of this operon, *sepL*. *sepL* encodes a protein composed of 351 amino acids (aa) with a predicted molecular weight of 39.95 kDa (Kresse *et al.*, 2000). Like many genes in the LEE it is highly conserved between EHEC and enteropathogenic *Escherichia coli* (EPEC) strains (93.7–94.3% identity). Original research in EHEC (Kresse *et al.*, 2000) indicated that SepL was a T3S regulator associated with the bacterial membrane and predominately detected in the outer membrane fraction. Further research indicated that EPEC SepL is either in the cytoplasm (O'Connell *et al.*, 2004) or in both the cytoplasm and the bacterial membrane (Deng *et al.*, 2005). As shown by several studies, SepL is crucial for the export of EspADB but also essential for controlling T3S effector secretion (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Deng *et al.*, 2004, Deng *et al.*, 2005). How SepL works to govern this switch is not known. Recent research by Deng *et al.*, (2004) revealed that another protein, SepD, might be part of this SepL switch as well. It has been demonstrated by several research groups that deleting *sepD* results in a similar phenotype to knocking out *sepL* (Deng *et al.*, 2004). It was shown that SepD is a binding partner of SepL with protein interactions investigated using both yeast two-hybrid and standard *in vitro* approaches (Creasey *et al.*, 2003b, O'Connell *et al.*, 2004). Deletion of either *sepL* or *sepD* leads to an increase in the levels of secreted Tir and other effector proteins and this increased effector protein secretion is not considered to be controlled at the transcriptional level (Deng *et al.*, 2004, Deng *et al.*, 2005). However, it is unclear how SepL/SepD controls effector secretion although a recent report showed that wild type EHEC/EPEC/CR can also display a Δ*sepL* or Δ*sepD*-

like phenotype when cultured under conditions of low calcium (Deng *et al.*, 2005). Therefore, SepL and SepD were proposed to act as a gate to allow translocator export and this gate dissociates or changes to allow effectors to be exported in response to a drop in calcium levels following the opening of a conduit to the host cell (Deng *et al.*, 2005). However, there is no evidence that SepL or SepD interacts directly with the translocon proteins EspA, D or B. It was suggested in the original SepL study that SepL had DNA and/or RNA binding capacities as a putative nucleotide-binding domain was detected in the SepL protein sequence (Kresse *et al.*, 2000). However, no binding was detected between SepL and the *espADB* sequence (Kresse *et al.*, 2000).

As a YopN/TyeA family protein, SepL homologues are present in many T3SS pathogens (SsaL in *Salmonella*; YopN/TyeA in *Yersinia*; MxiC in *Shigella*; HrpJ in *Erwinia* and PopN in *Psudomonas*) and all of them have been shown to be important for T3 secretion control, although the phenotypes and controlling mechanisms were varied (Ferracci *et al.*, 2005, Coombes *et al.*, 2004, Botteaux *et al.*, 2009, Nissinen *et al.*, 2007, Yang *et al.*, 2007). As TyeA controls the secretion of specific effector proteins in *Yersinia* spp. (Cheng and Schneewind, 2000b, Day *et al.*, 2003, Sundberg and Forsberg, 2003). it was decided to investigate full-length and carboxy-terminal truncates (TyeA homologue) of SepL in terms of the known activities of SepL including localization, SepD binding, translocon export and Tir secretion control.

3.2 Results

SepL is homologous to a combination of YopN and TyeA from *Yersinia* spp. (Pallen *et al.*, 2005a) (Fig. 3.1A). The carboxy terminus of SepL (final 83 aa) is homologous with TyeA and so carboxy-terminal truncates of SepL fused to GFP were tested to determine if these deletions can separate the different functions of SepL. The functions analysed were the capacity to: (i) localize to the bacterial membrane, (ii) bind to SepD, (iii) restore EspD secretion in *sepL* mutants and (iv) reduce Tir secretion in *sepL* mutants. Five truncated proteins were initially constructed as illustrated in Fig. 3.1A.

3.2.1 Localisation of SepL

Previous research has demonstrated that SepL in EHEC O157 can be detected in both membrane and cytoplasmic fractions but is not secreted into growth media. It was also found SepL has a binding partner-SepD which in a *sepD* deletion strain results in a Δ*sepL* like hypersecretion phenotype (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Creasey *et al.*, 2003b). The truncated fusion constructs (Fig. 3.1A) were examined for their presence in bacterial membrane-containing fractions. The full-length SepL–GFP construct was detected in a membrane-enriched fraction of bacteria prepared from T3S-permissive conditions while this was not the case for GFP alone, which was only detectable in the cytoplasmic fraction (Fig. 3.1B). Two other SepL fusions (N-terminal 340 aa and 290 aa SepL proteins fused to GFP) were detected in the membrane-enriched fractions, although this distribution was prevented by any further truncation of SepL with smaller fusions only being detectable in the cytoplasmic fraction. Membrane protein-OmpA and cytoplasmic protein-GroEL were used as controls in this study. The

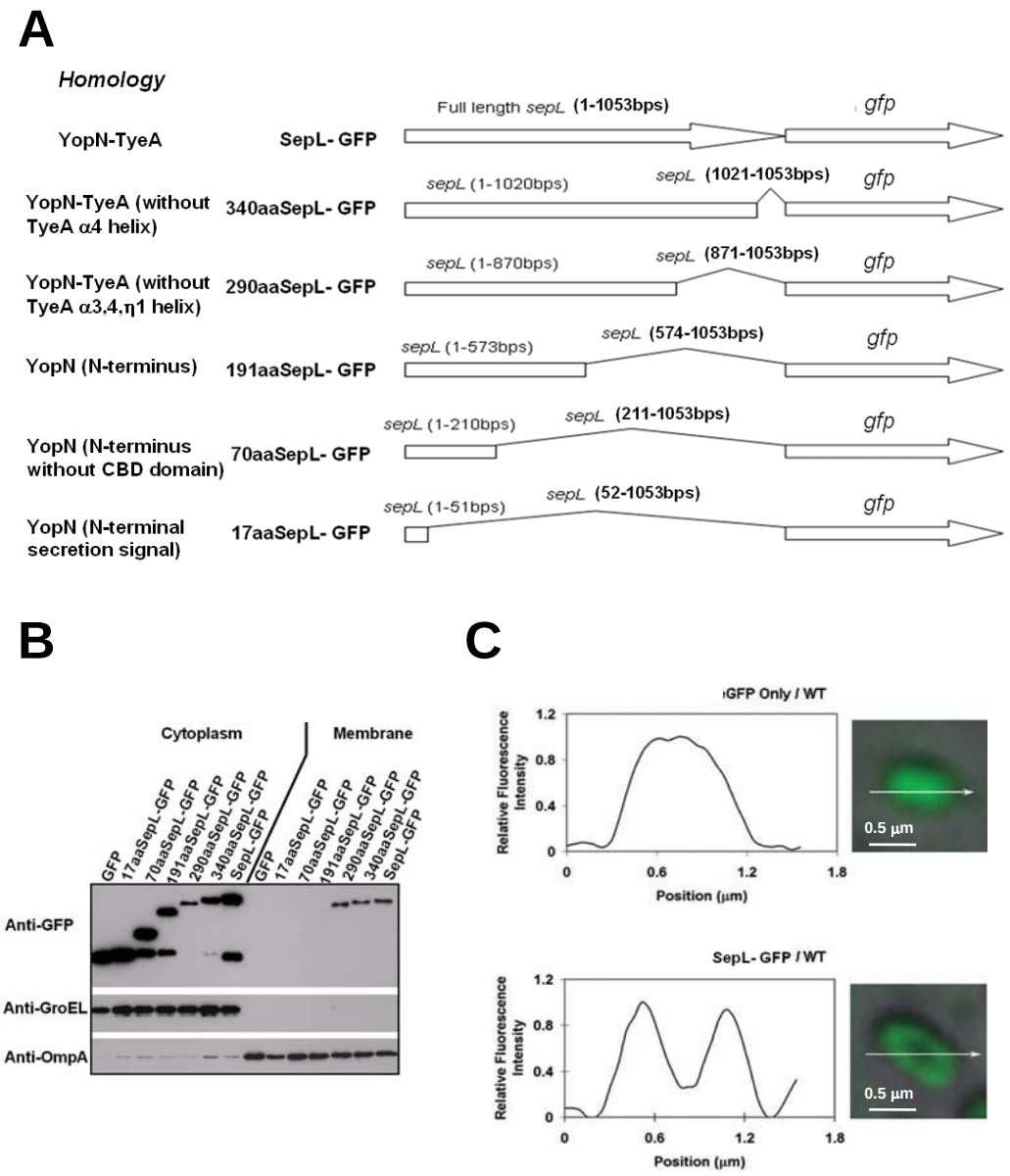


Fig. 3.1. Analysis of SepL–GFP localization.
A. Figure of the SepL truncates fused to GFP that were used in the study. The full-length, 340 aa, 290 aa and 267 aa SepL regions were also generated as carboxy-terminal 6x His-tagged constructs.
B. Membrane localization of SepL truncates fused to GFP. Western blotting was used to detect GFP as described in Materials and Methods. Integrity of the fractions was confirmed with anti-GroEL and anti-OmpA antibodies.
C. Fluorescence and phase contrast image overlays demonstrating localization of GFP and SepL–GFP in a single bacterium. Fluorescence levels were measured along a transect drawn through individual cells. The examples shown demonstrate the peripheral localization of SepL–GFP but not GFP alone.

distributions of OmpA and GroEL in the same samples were as expected (OmpA > 90% in the membrane-enriched fraction; GroEL > 99% in the cytoplasmic fraction) (Fig. 3.1B). SepL-GFP was also observed by fluorescence microscopy and imaging of individual bacteria containing the full-length SepL–GFP clearly showed a higher concentration of fluorescence localized to the periphery of the bacteria (Fig. 3.1C). This was not the case for bacteria expressing just GFP (Fig. 3.1C). The Western blots and single cell images both indicated that SepL localizes to the bacterial membrane and this association does not require the carboxy-terminal 61 aa of SepL.

As reported before, SepD is a binding partner of SepL. A logical question was what effect would *sepD* deletion have on the localization of SepL. *sepD* was deleted by allelic exchange and the construct confirmed by complementation with *sepD* on a plasmid (pDW20) (Fig. 3.2). Localization of SepL–GFP was detected using a combination of protein immunoblotting and fluorescence microscopy (Figs 3.3A and B and 3.1C). In the membrane-containing fraction, the proportion of the full-length SepL–GFP hybrid protein was much reduced in a *sepD* background (Fig. 3.3A). In addition, in this background, the localization of SepL–GFP was clearly different in the bacteria. The distribution now appeared asymmetric (Fig. 3.3B), unlike the even distribution of GFP alone, indicating that in the absence of SepD, SepL may associate with another cellular protein that exhibits this asymmetric distribution. The different length SepL-GFP fusions were then examined for their capacity to bind to purified GST–SepD attached to a glutathione-sepharose 4B column.

Full-length SepL as well as the 340 aa and 290 aa fusions bound to SepD but SepD interaction with further truncation from the carboxy terminus was not

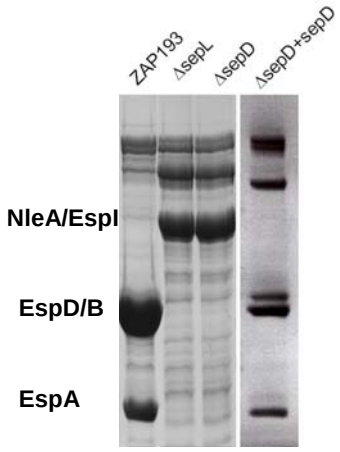


Fig. 3.2. Secretion profiles of EHEC wild type strain, Δ*sepL*, Δ*sepD* and Δ*sepD* complimented with a SepD plasmid (pDW20). The secretion of proteins into the bacterial culture supernatant was prepared as described in M&M. Protein samples were separated by SDS-PAGE and subsequent Colloidal blue staining.

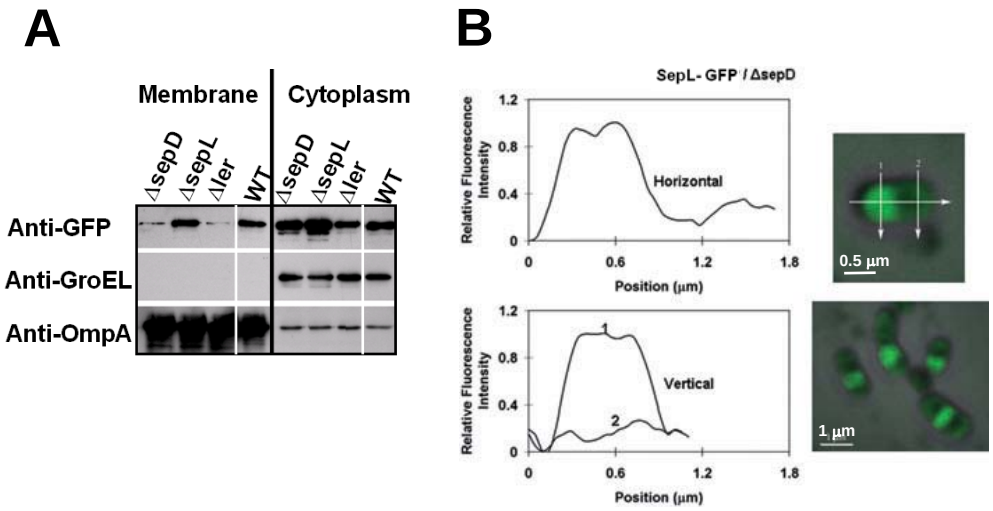


Fig. 3.3. SepL–GFP localization in different EHEC O157 genetic backgrounds.
A. Western blot detection of GFP in membrane and cytoplasmic fractions of the described EHEC O157 strains expressing SepL–GFP (pDW6). Western blotting was carried out as described in Materials and Methods.
B. Localization of SepL–eGFP in a *sepD* mutant. Fluorescence intensities across a representative bacterium expressing the SepL–GFP fusion are shown. The asymmetric distribution of the SepL–GFP fusion in a *sepD* mutant background (ZAP1144) is apparent when compared with the distribution in the wild-type background (Fig. 3.1C).

detected (Fig. 3.4). The data indicates that the SepL truncates that localized to the membrane are the same as those that bound to SepD. Given that deletion of *sepD* also reduced membrane association of SepL and altered its cellular distribution, it is evident that SepD is responsible for membrane localization of SepL.

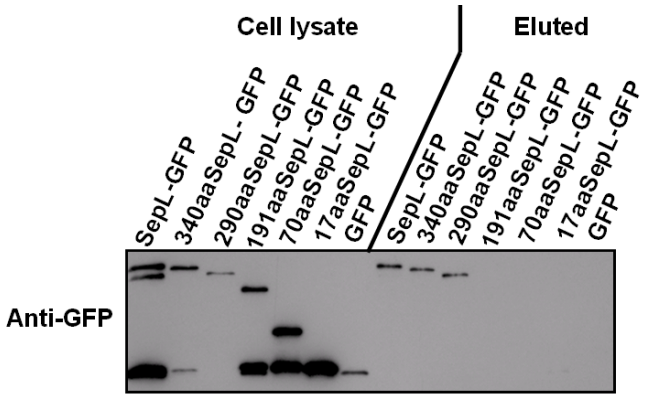


Fig. 3.4. Capacity of SepL truncates to bind to SepD. Each of the SepL–GFP constructs was tested for the capacity to bind to immobilized GST–SepD. Following elution, the SepL–GFP truncates were detected by Western blotting as described in Materials and Methods.

3.2.2 Co-localization of SepL and EspA filaments

The surface production of EspA filaments has been previously investigated by our research group. From the results it was evident that only a subset bacterial cells had EspA filaments on their surface (Roe *et al.*, 2003b). It was therefore interesting that when bacteria containing the full length SepL-GFP fusion were examined by fluorescence microscopy, GFP expression was only observed in a subset of bacteria when cultured under the T3S stimulating conditions used. Co-staining of EspA filaments with SepL-GFP expression showed that there was a positive correlation between SepL-GFP expression and EspA production (Chapter 2: Fig. 2.2). From the fluorescent image, only the bacterial cell with highly expressed SepL-GFP could produce EspA filaments on the surface. This result also supports that SepL protein is crucial for translocon proteins export.

Although it was very interesting why SepL-GFP expression can only be observed in certain cells which produced EspA filaments, I was investigating where SepL was located inside bacterial cells. It was reported that SepL

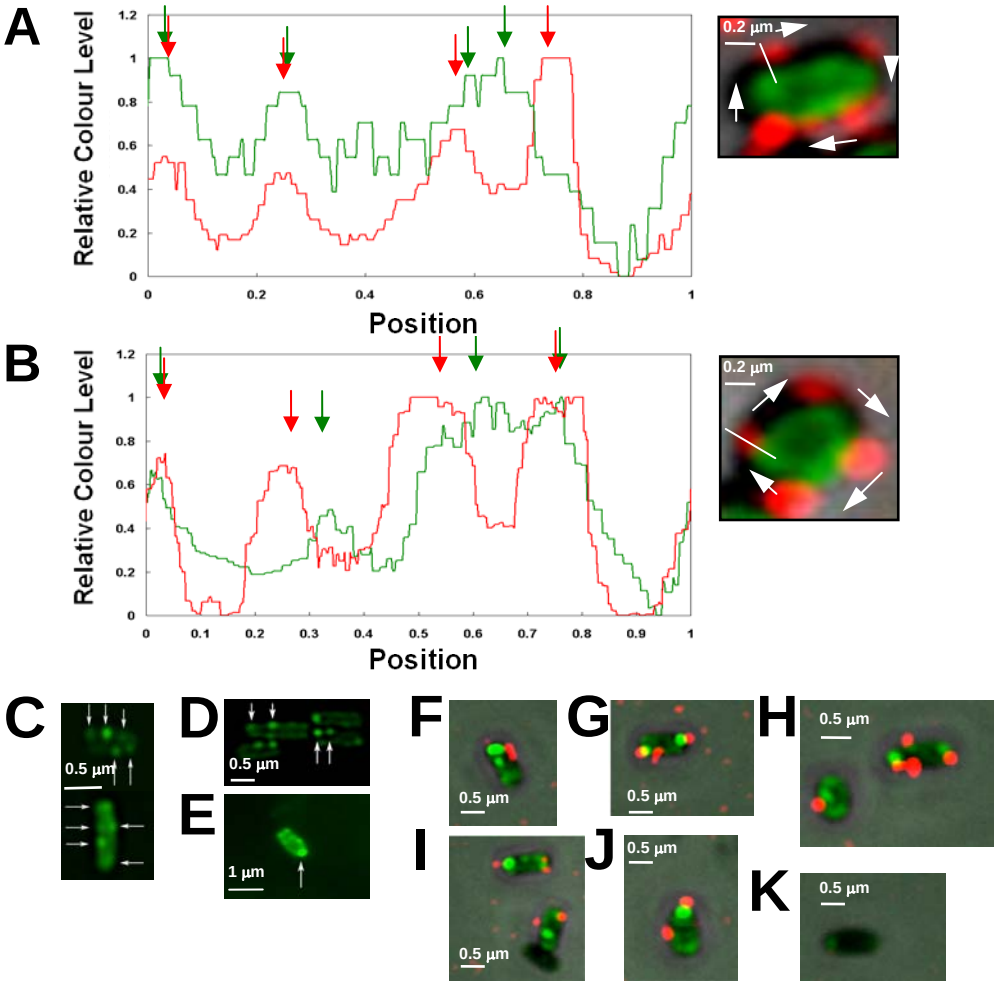


Fig. 3.5. Localisation of SepL-GFP in EHEC O157 and association with EspA filaments. (A-B) Images of SepL-GFP fluorescence and EspA filament expression (detected following immuno-staining) overlaid onto the relevant phase contrast image. EspA filamentation was localised by Alexa Fluor 594 emission (617 nm) from the secondary antibody (Invitrogen) following immuno-staining. Best fit ellipses were drawn around the bacterial cells that dissected the main fluorescence foci and levels were determined along this line in the direction of the arrows starting from the white line indicated. This process was repeated for smaller best fit ellipses that dissected the main GFP fluorescent foci (510nm). The fluorescence emission values were plotted over the length of the ellipses between 0 (start) and 1 (end) and the main fluorescence foci were indicated by arrows (Green: SepL-GFP; Red: EspA). The graphs demonstrate an association between the position of EspA filaments and membrane-associated foci of SepL-GFP fluorescence with each of the apparent EspA filaments registering with a SepL-GFP focal point to within 100 nm. (C-E) Fluorescent foci were more evident when EHEC O157 expressing the SepL-GFP (pDW6) was co-incubated with EBL (embryonic bovine lung) cells. (C) Foci, indicated by the arrows, were apparent 2 hr following infection and were more marked at 4 hr (D). At six hours larger foci were detected at the poles of the bacteria (E). (F-K) The corresponding localization of EspA (Red) and SepL-GFP (Green) is also very clear in EHEC when bacteria were incubated with EBL cells. Imaging and measurements were performed as described in Materials and Methods.

protein of EPEC only existed in the bacterial cytoplasm by O'Connell *et al.* 2004, while other groups suggested that SepL protein was not only present in the bacterial cytoplasm but also associated with the bacterial membrane. In order to investigate these contradictory results for SepL localization, both biochemical methods and fluorescence microscopy were employed to localize SepL protein in EHEC O157:H7. As shown above, it is clear that SepL is not only detected in the bacterial cytoplasm but also in membrane-containing fractions. Further examination of individual cells revealed fluorescence foci at the periphery of the bacterium. Immuno-fluorescence staining of EspA filaments indicated that the position of SepL-GFP foci corresponded with the position of extracellular EspA filaments. To confirm this, GFP (SepL) and Alexifluor 594 (immuno-staining of EspA) fluorescence levels around bacterial cells was determined. Example plots are shown in Fig. 3.5A-B. This type of analysis indicates that SepL-GFP is present at higher concentrations inside the cell at sites engaged in EspA secretion, presumably representing the membrane spanning type 3 secretion apparatus. As expected, precise co-localization was not observed as EspA filaments are a surface structure and SepL is a membrane-associated protein only detectable inside the bacterial cell. In order to examine the localisation of SepL-GFP during A/E lesion formation on eukaryotic cells, fluorescence microscopy was carried out on bacterial cells expressing the SepL-GFP following addition to embryonic bovine lung (EBL) cells (Fig. 3.5 C-K). At two hours following infection, SepL-GFP expression was detected and weak foci imaged (Fig. 3.5C), these foci are clearly distributed around the edge of the bacteria at four hours following infection (Fig. 3.5D). After six hours, some of the fluorescent foci become distributed to the bacterial poles (Fig. 3.5E).

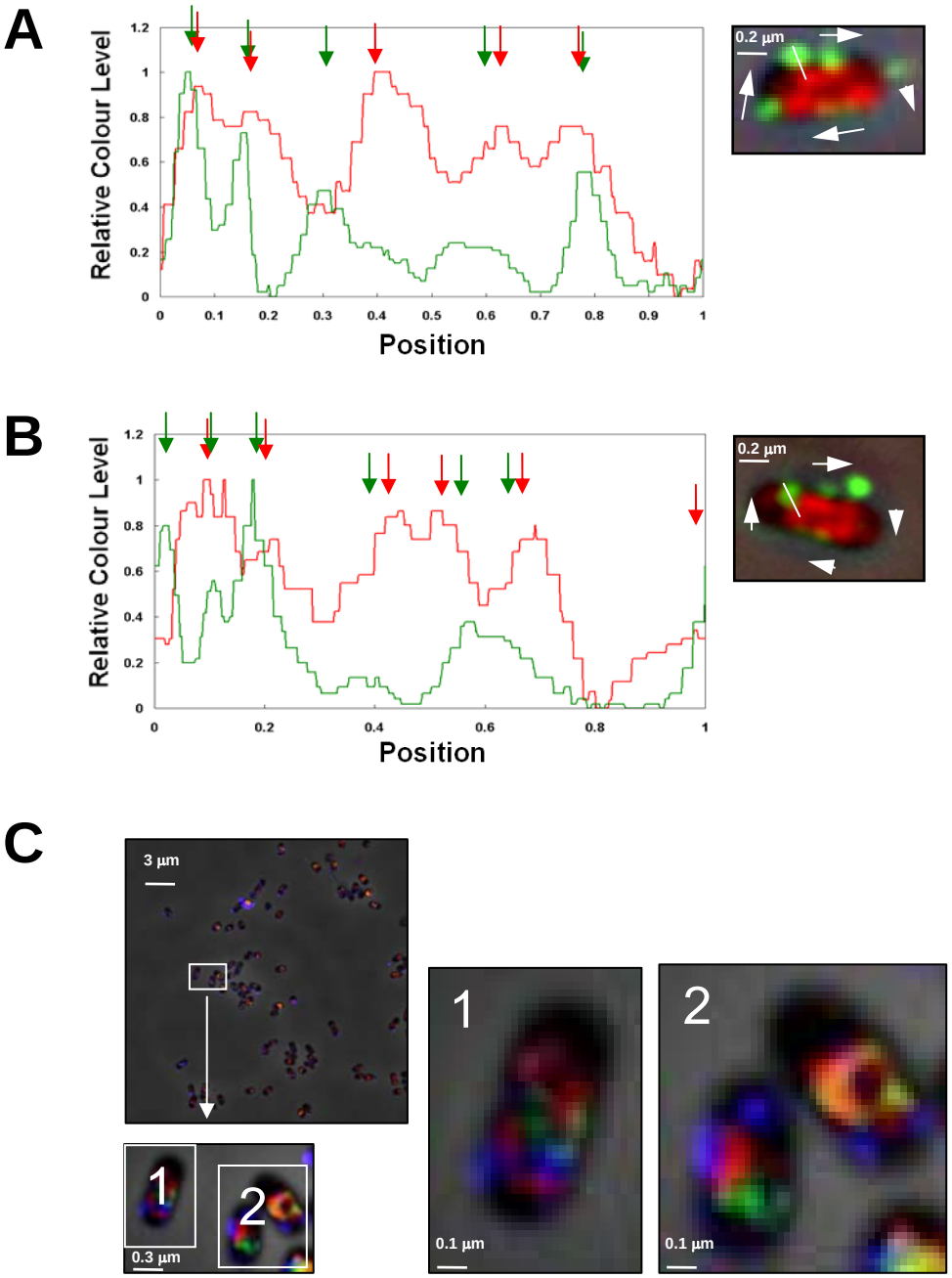


Fig. 3.6. Co-localization of SepL, SepD and EspA
Localisation of SepD-RFP in EHEC O157 and association with EspA filaments (A & B). Overlay images of SepD-RFP (583 nm emission peak, coloured red) and immuno-stained EspA filaments (FITC-conjugated secondary antibody, 518 nm emission peak, coloured green) on a phase contrast background. The two fluorescence signals were measured along best fit ellipses hand drawn around and within individual bacterial cells as described for Fig. 3.5 and the main fluorescence foci on the plots were indicated by arrows (Green: EspA; Red: SepD-RFP) . The graphs demonstrate that the position of EspA filaments register with the SepD-RFP foci to within 100 nm. (C) Co-localization of SepL-GFP (green), SepD-RFP (Red) and EspA (Blue) was visualized as described in Chapter 5. Although the overlap of SepL/SepD is obvious (C2), SepD is more likely membrane associated (C1-2).

3.2.3 Localisation of SepD and association with SepL

Previous work has demonstrated an *in vitro* interaction between SepL and SepD and our localisation studies above demonstrate that SepD is required for SepL localisation and expression. To characterise this interaction further in the individual bacteria a fusion of SepD to red fluorescent protein (RFP: Sorensen *et al.*, 2003) was constructed. The localisation of this fusion was determined in bacteria immuno-stained for EspA filaments. SepD-RFP localised to internal foci corresponding to the external position of EspA filaments (Fig. 3.6A-B). Compared with SepL the majority of the fusion protein was present in foci rather than distributed generally in the bacterial membrane. Localisation of SepL, SepD and EspA filaments in single cells was attempted by integrating a single copy of the SepL-GFP fusion into the bacterial chromosome and use of the SepD-RFP construct with Alexa-fluor 405 blue immuno-staining of EspA filaments. As expected, there was a close association exhibited among SepL-GFP, SepD-RFP and EspA filament location (Fig. 3.6C). Due to different vision angles, co-localization of SepL-GFP/SepD-RFP was presented as mixed proteins (Fig. 3.6C2) or proteins located next to each other (Fig. 3.6C1)when imaged under fluorescence microscope. Although the overlap of SepL/SepD locations was observed clearly (Fig. 3.6C2), it is also noticed that SepD is more likely to be membrane associated as suggested by fluorescence images (Fig. 3.6C1-2).

3.2.4 Complementation of translocon (EspD) export in a sepL mutant

Translocon (EspADB) export was stopped in a *sepL* mutant and it can be complemented by supplying *sepL in trans* (Kresse *et al.*, 2000). In this study, full-length SepL was fused to 6x histidine or GFP and both fusions could

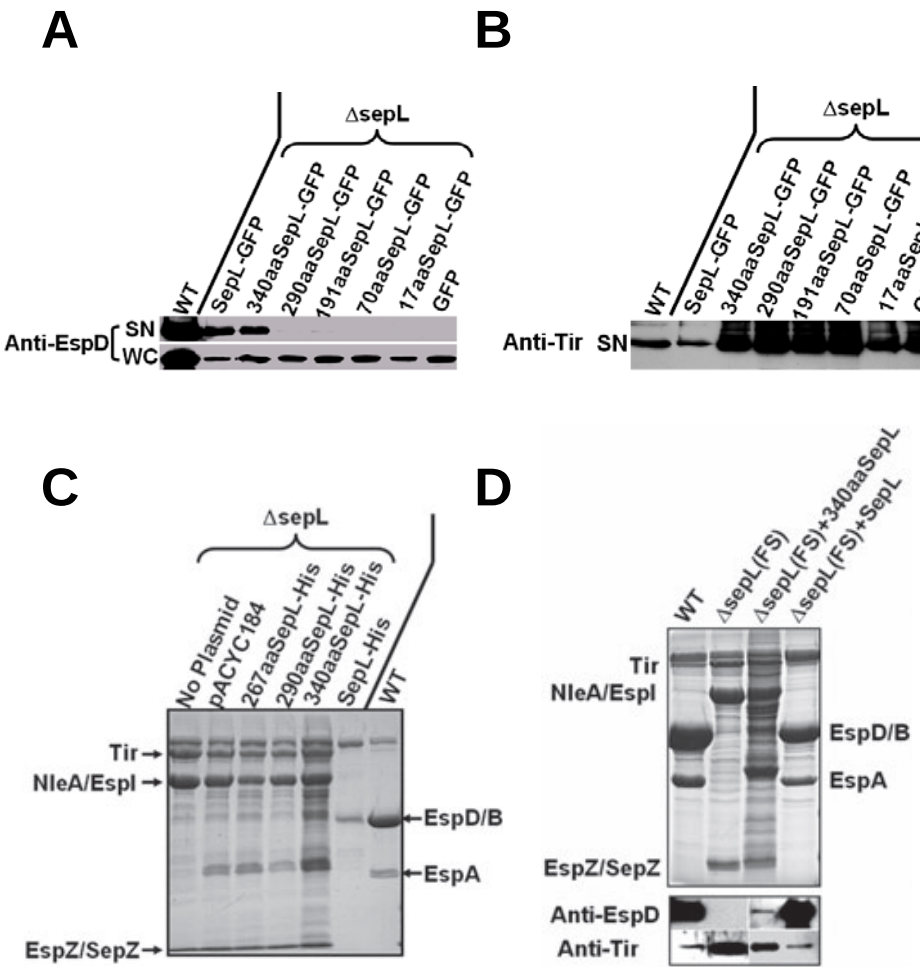


Fig. 3.7. Analysis of protein secretion in *E. coli* O157 (Δ*sepL*) expressing different SepL truncates.
A. The first two panels show EspD levels in the supernatant (SN) and whole-cell fractions (WC) when the different SepL–GFP constructs were used to complement a *sepL* mutant (ZAP1143). B. The panel shows detection of secreted Tir by Western blotting from the samples labeled in (A). C. Analysis of bacterial supernatants from a *sepL* mutant complemented with the His-tagged SepL constructs described in the Fig. 2.1 and the text. The wild-type (ZAP193) supernatant profile is also shown for comparison. These experiments confirm the results obtained with the SepL–GFP constructs in (A) and (B) and demonstrate that the carboxy-terminal 11 aa of SepL are required to limit the secretion of effector proteins in addition to Tir. D. Colloidal blue staining of secreted proteins from *E. coli* O157 engineered to contain a frameshift mutation in *sepL* (ZAP1211) and then complemented with full-length SepL (pDW48) and the C-terminal 11 aa deletion of SepL (pDW47). Western blotting for EspD and Tir for these strains is also shown. Preparation of protein samples and detection of Tir and EspD by Western blotting were as described in Materials and Methods.

complement a complete *sepL* deletion for EspD secretion to a similar extent as complementation with untagged SepL (Fig. 3.7A and C). A *sepL* deletion strain, ZAP1143, was used in our study and complementation of this never

achieved the EspD secretion levels of the wild-type strain and this was also the case for complementation with *sepL* alone, pDW24 (Fig. 3.7 A, C and D). Analysis of the SepL truncates in the *sepL* deletion strain indicated that only the full length protein and the 11 aa carboxy-terminal deletion (-11) were able to export EspD. Deletion of 11 aa from SepL partially complemented the *sepL* deletion for EspD secretion (Fig. 3.7A). It was interesting to note that a deletion of 61 aa (leaving a 290 aa SepL derivative) failed to export EspD despite membrane localization and SepD binding activity. It is suggested that the failure to completely complement a full *sepL* deletion is due to changes in the LEE4 transcript as discussed in chapter 2. To verify the phenotypes of the SepL truncates in a *sepL*-mutated background, a frameshift mutation was constructed in *sepL* (ZAP1211) that will have less impact on the structure of the LEE4 transcript by inserting a single base into the *sepL* ORF. In this background, EspD secretion could be complemented completely by *sepL in trans* (Fig. 3.7D). The 11 aa carboxy-terminal deletion in this background was still able to secrete EspD protein into liquid culture media but at reduced levels compared with the full-length complement (Fig. 3.7D).

3.2.5 Regulation of effector protein secretion

A *sepL* mutant is characterized by high levels of Tir secretion (Kresse *et al.*, 2000)(Fig. 3.7B) and of other effector proteins including NleA (Deng *et al.*, 2004). To investigate the function of the different SepL truncates, the levels of Tir secreted were determined by Western blotting in the *sepL* mutants transformed with the different SepL fusions. Of note was that only the full-length SepL construct was able to lower Tir secretion levels to those

demonstrated for the wild-type strain (Fig. 3.7B). To confirm this result and to rule out any impact of GFP on the phenotypes, four similar SepL truncates (His-tagged) were made and also tested. These were: (i) full-length SepL, (ii) a protein with the first 340 aa of SepL but deleted for the carboxy-terminal 11 aa, (iii) the first 290 aa of SepL but deleted for the carboxy-terminal 61 aa and (iv) the first 267 aa of SepL but deleted for the carboxy-terminal 84 aa. These variants had exactly the same phenotypes as the respective GFP fusions in the *sepL* deletion background. For example, analysis of the general secretion profiles indicated that the 11 aa carboxy-terminal SepL truncate failed to control secretion of Tir, whereas complementation with full-length SepL could (Fig. 3.7B-C). Moreover, it was apparent that this regulation also applied to other T3S effectors with sizes equivalent to those of NleA and EspZ as shown by SDS-PAGE (Fig. 3.7C). This result was also confirmed in the *sepL* frameshift mutant (ZAP1211) background (Fig. 3.7D).

3.2.6 Tir binds to the carboxy terminus of SepL

As effector protein secretion is limited by full length SepL in EHEC O157 strain, an assay was carried out to examine the direct interaction between SepL and effector proteins. The over-secreted effector proteins from a *sepL* mutant were separated by SDS-PAGE and Far-Western immunoblotting carried out to examine their interaction with 6x His-tagged SepL. As shown in the blot (Fig 3.8A), SepL only interacted clearly with one protein in the bacterial supernatant and this protein was of a molecular weight equivalent to Tir. This interaction was confirmed using a GST pull down experiment. Immobilized GST–SepL was incubated with 6x His-tagged Tir and Tir-His was found to be eluted with GST-SepL (Fig. 3.8B). As a control, His-tagged

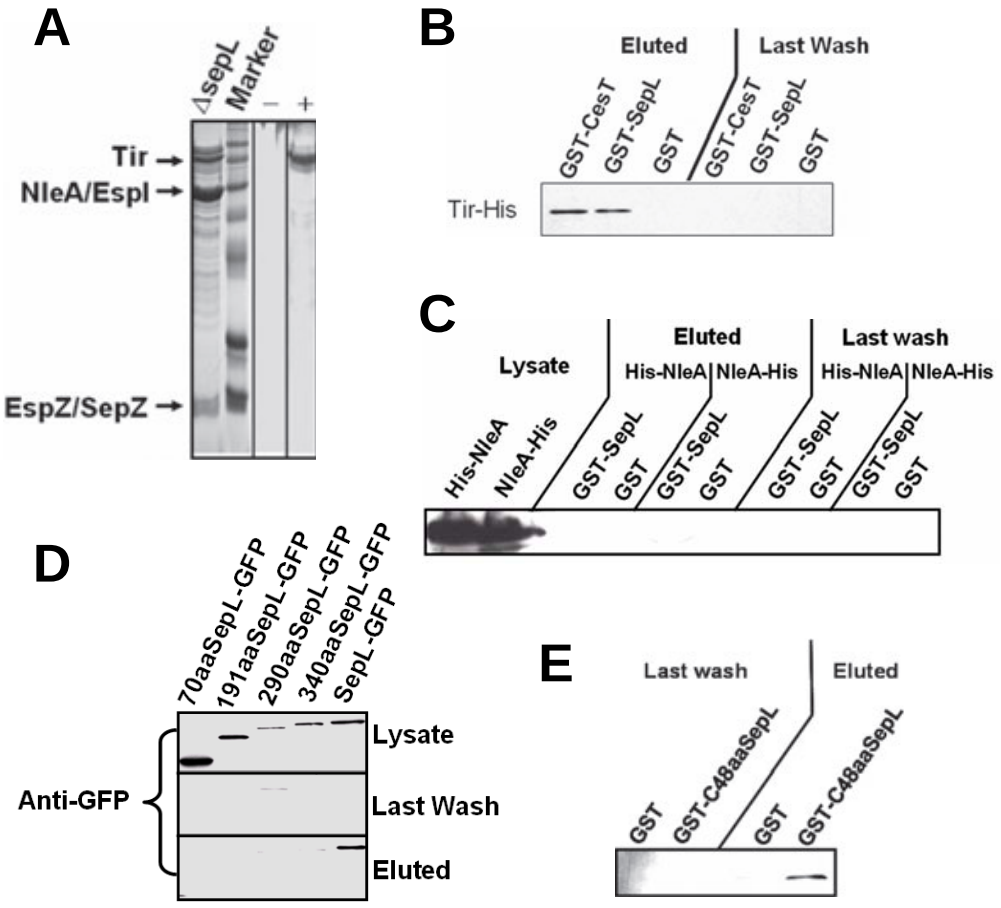


Fig. 3.8. SepL binds to Tir. A. Detection of supernatant proteins that bind to SepL. Supernatant proteins from a *sepL* mutant (ZAP1143) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with SepL-His (+) prepared from *E. coli* BL21. As a control (-), the incubation with SepL-His was omitted. The Far-Western was developed following incubation with an anti-penta-His antibody as described in Materials and Methods. B. Detection of Tir binding by immobilized SepL. His-tagged Tir was prepared in *E. coli* BL21 and incubated with immobilized GST–SepL, GST–CesT and GST alone. Following elution, Tir-His was detected by Western blotting. C. NleA was expressed either with a C-terminus 6 x histidine tag or an N terminus 6 x histidine tag. Both were expressed and detectable in *E. coli* BL21 lysates (first two lanes). The lysates were run through columns containing immobilised GST or GST-SepL. The columns were then washed three times with PBS and then eluted with GST elution buffer. The fusion proteins were detected as described in Materials and Methods. There was no evidence of an interaction between SepL and NleA in contrast to the binding of Tir by SepL (B).
D. The C-terminus of SepL is required for Tir binding. 6x His-tagged Tir was purified on nickel-NTA columns and *E. coli* K-12 (AAEC185) lysates, containing the different indicated truncates of SepL fused to GFP, were run through the columns. Following washes, proteins were eluted and separated by PAGE. SepL constructs were then detected by Western blotting using an anti-GFP antibody. E. The carboxy terminus of SepL is sufficient to bind to Tir. The C-terminal 48 aa of SepL was fused to GST and immobilized onto a column. 6x His-tagged Tir bound to the 48 aa C-terminal SepL construct and was detected in the eluate by Western blotting.

Tir bound to GST–CesT (+ve control) and GST–SepL but not to GST (-ve control) alone. As the -11 SepL truncate failed to limit Tir secretion, it was anticipated that the SepL interaction with Tir requires the final 11aa carboxy terminus of SepL. This was confirmed as 6xHis-tagged Tir was shown to bind to full-length SepL–GFP but not to any of the SepL truncates (Fig. 3.8C). Therefore, the next question was if the carboxy terminus of SepL alone could interact with Tir. A comparative domain analysis with YopN/TyeA (Fig. 3.12) was carried out to select a region of SepL for testing its capacity of Tir binding. The carboxyl-terminus (48aa) was fused to GST and immobilized on a Glutathione-Sepharose 4B column. Tir-His was able to bind to this hybrid protein but not to GST alone (Fig. 3.8E). The Far-Western analysis indicated that Tir was the only protein detected interacting with SepL from the different over-secreted effector proteins. However, this may be a result of misfolded effector proteins which do not expose an interaction domain after being transferred onto the nitrocellulose membrane. To determine if SepL could interact with another secreted effector protein, NleA was tagged with either an amino- or carboxy-terminal 6x histidine and used to examine a possible interaction. Neither fusion was found to bind to SepL using a GST-pull down assay (Fig. 3.8C). Therefore, in this study only Tir is pulled out as a SepL binding target among all the hypersecreted proteins although SepL could restrict the secretion of other effectors. A Tir binding domain was mapped to the carboxyl-end of SepL. The final 48 aa of SepL are sufficient to interact with Tir.

3.2.7 Analysis of Tir domains that interact with SepL and CesT

In the cytoplasm, Tir is stabilized by its chaperone-CesT that is also required

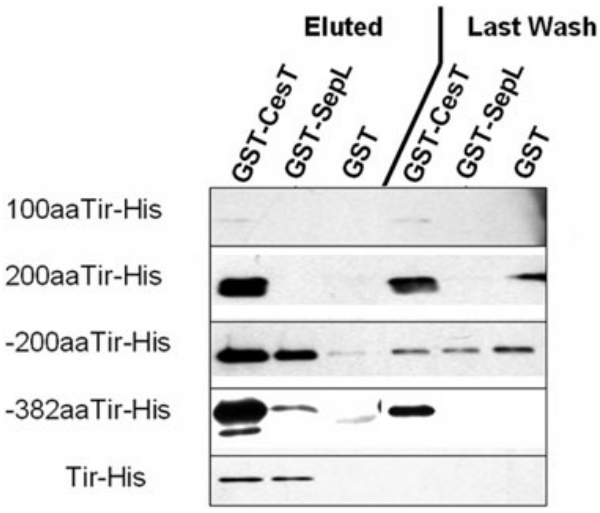


Fig. 3.9. SepL and CesT binding domains of Tir.
Different 6x His-tagged truncates of Tir were incubated with immobilized GST–CesT, GST–SepL and GST alone. Following elution, 6x His-tagged Tir constructs were detected as described in Materials and Methods. 100aaTir-His and 200aaTir-His are His-tagged constructs containing the first 100 and 200 aa of Tir respectively. The first 200 aa of Tir is known to contain a CesT binding region (Abe *et al*., 1999; Elliott *et al*., 1999). -200aaTir-His and -382aaTir-His are His-tagged constructs containing Tir without the first 200 and 382 aa respectively.

for efficient secretion of Tir *via* a functional T3SS (Abe *et al.*, 1999, Elliott *et al.*, 1999a). It is known that CesT is also an essential chaperone for the secretion of many other hyper-secreted effector proteins (Thomas *et al.*, 2005). Tir secretion may be limited via SepL binding to Tir but as a multi-function chaperone, the role of CesT in this interaction is unknown. To determine SepL and CesT binding domains in Tir, different Tir truncates were labeled by 6x histidine and binding capacities to immobilized GST–SepL, GST–CesT and GST alone were tested (Fig. 3.9). It has been reported that CesT was able to bind to the first 233 aa of Tir (Abe *et al.*, 1999, Elliott *et al.*, 1999a). My results demonstrated that not only did the N-terminal 200 aa of Tir bind to CesT but another CesT binding domain was mapped in the carboxyl-terminus of Tir (Fig. 3.9). As suggested by the results described

above, deletion of the first 200 and 382 aa of Tir still produced a polypeptide that could bind to CesT. By contrast, the first 200 aa of Tir did not interact with SepL but the remainder of the protein did bind to SepL as did the 382 amino-terminal truncate. The data indicate that there are at least two regions in Tir that can interact with CesT and that one of these might compete with SepL–Tir binding. Further mapping of these interactions would be required to confirm this.

3.2.8 The interaction of Tir with SepL controls the timing of secretion

As an 11 aa deletion of SepL retains the capacity to export translocon proteins but is unable to limit effector protein secretion, it raised the possibility that effector protein export was now occurring at the same time as translocon export as the capacity of SepL to bind Tir potentially sequesters Tir export and somehow limits the secretion of other effector proteins during translocon assembly. To test this, the timing of EspD and Tir secretion was analyzed in the wild-type strain and a *sepL* mutant complemented with either full-length *sepL* or the 11 aa carboxy-terminal deletion (-11). T3S activation in different EHEC strains was synchronized using a medium shift for which bacteria were cultured initially in a medium (LB) that is not permissive for T3S and then transferred to a medium (MEM-Hepes) that induces T3S. Following the transition, samples were taken at regular intervals and the levels of secreted Tir and EspD determined as described in Materials and Methods. For the full-length SepL complement in the *sepL* deletion, EspD secretion was detectable but not Tir at early time points (Fig. 3.10A). This pattern was similar in the wild-type strain (Fig. 3.10A) By contrast, in the *sepL* mutant complemented with the C-terminal 11 aa deletion of SepL, Tir

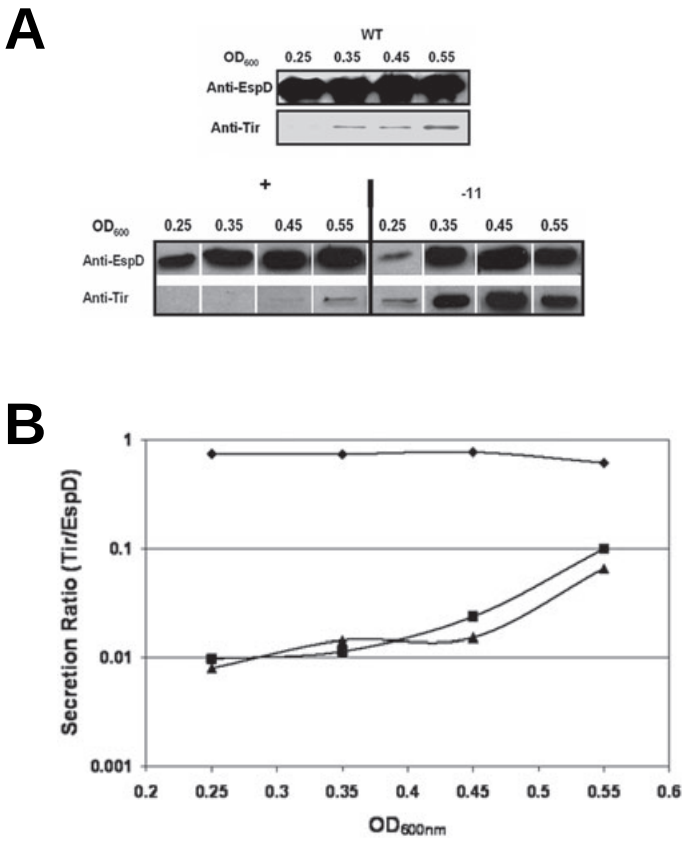


Fig. 3.10. Secretion timing is altered by deletion of the carboxy terminus of SepL.
A. *E. coli* O157:H7 (ZAP193) (top panel) and the *sepL* deletion (ZAP1143) (second panel) complemented by either full-length SepL (pDW6) or SepL with a deletion of the final 11 aa (pDW30) were cultured in LB that represses T3S and then transferred into MEM-HEPES that induces T3S.
B. Samples were taken at defined optical densities and the levels of secreted EspD and Tir determined as described in Materials and Methods. The cultures were repeated in triplicate and the blots shown represent the secretion patterns from one set from which the ratio of secreted Tir to EspD is also shown. Wild type, ZAP193 (▲); Δ*sepL*, ZAP1143 complemented with full-length SepL (■), or with the C-terminal -11 aa truncate (◆).

secretion was detectable along with EspD secretion at early time points (Fig. 3.10A) following the induction of T3S. While it is appreciated that Tir secretion levels are higher in the truncate-complemented background it is clear from analysis of the EspD/Tir secretion ratios (Fig. 3.10B) that Tir secretion is no longer delayed in the *sepL* mutant complemented with the 11 aa truncate by comparison with full-length *sepL* complementation or the wild type. Consequently, secretion hierarchy is disrupted when the capacity of SepL to bind Tir is removed.

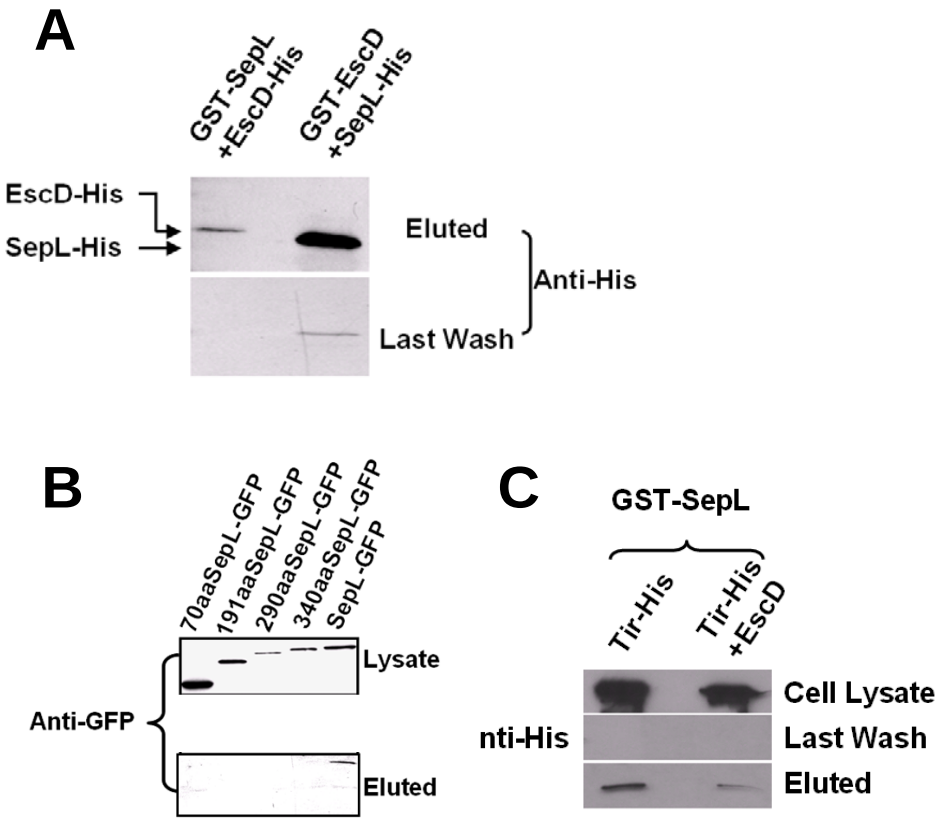


Fig. 3.11. SepL binds to EscD
A. 6 x Histidine-tagged EscD was pulled down by GST-SepL (left hand side) and 6 x Histidine-tagged SepL was pulled down by GST-EscD (right hand side); B. The C-terminus of SepL is required for EscD binding. GST-tagged EscD was purified on Glutathione Sepharose 4B columns and *E. coli* K-12 (AAEC185) lysates, containing the different indicated truncates of SepL fused to GFP, were run through the columns. Following washes, proteins were eluted and separated by PAGE. SepL constructs were then detected by Western blotting using an anti-GFP antibody. C. Competitive interactions between SepL, EscD and Tir. GST-SepL was bound to G-Sepharose 4B beads, and then incubated with a lysate containing 6x Histidine-tagged Tir. Following washes with PBS, 6x Histidine-tagged Tir was eluted with GST-SepL. The amount of SepL bound to Tir was reduced when EscD was added to the lysate.

3.2.9 Tir and SepL interact with EscD

As Tir export is restricted by SepL binding there needs to be a mechanism to release Tir from SepL once translocon assembly is completed. Previous research has demonstrated that a component of the T3S basal apparatus, EscD (Pas), is able to bind to Tir (Kresse *et al.*, 1998) and when *escD* is expressed from a plasmid Tir is secreted at higher levels (Ogino *et al.*, 2006). However, no mechanism was proposed for this regulation. I hypothesised

that if SepL was also able to bind to EscD and if this required the same SepL domain as for Tir binding then the over-expression of EscD could release Tir sequestered by SepL leading to elevated levels of secreted Tir. To test this interaction GST-SepL was constructed and retained on a glutathione column and shown to bind to 6 x Histidine-tagged EscD (Fig. 3.11A). This was confirmed using reciprocally-labelled EscD and SepL (Fig. 3.11A). Moreover, only the full length SepL-GFP construct bound to GST-EscD (Fig. 3.11B). This demonstrated that full length SepL does bind to EscD and this interaction, as with the interaction with Tir, requires the carboxy terminal 11 amino acids of SepL. Competitive binding between the three proteins was then demonstrated, for example with EscD being able to inhibit the Tir-SepL interaction (Fig. 3.11C). A function of this interaction could be to release Tir from SepL following translocon assembly.

3.3 Discussion

SepL and SepD are known as important switches governing the T3S hierarchy in EHEC/EPEC/CR. From the current study, I propose that SepL switches T3S between the translocon and effector protein substrates by binding to Tir and through this sequestration prevent the secretion of Tir and other effector proteins while the translocon components are being exported and assembled. This activity requires the carboxy terminus of SepL and can be separated from other SepL phenotypes, including its membrane localization, SepD binding and translocon export.

The C-terminus of SepL shares some homology with TyeA and the N-terminus of SepL some homology with YopN, both from *Yersinia* spp.

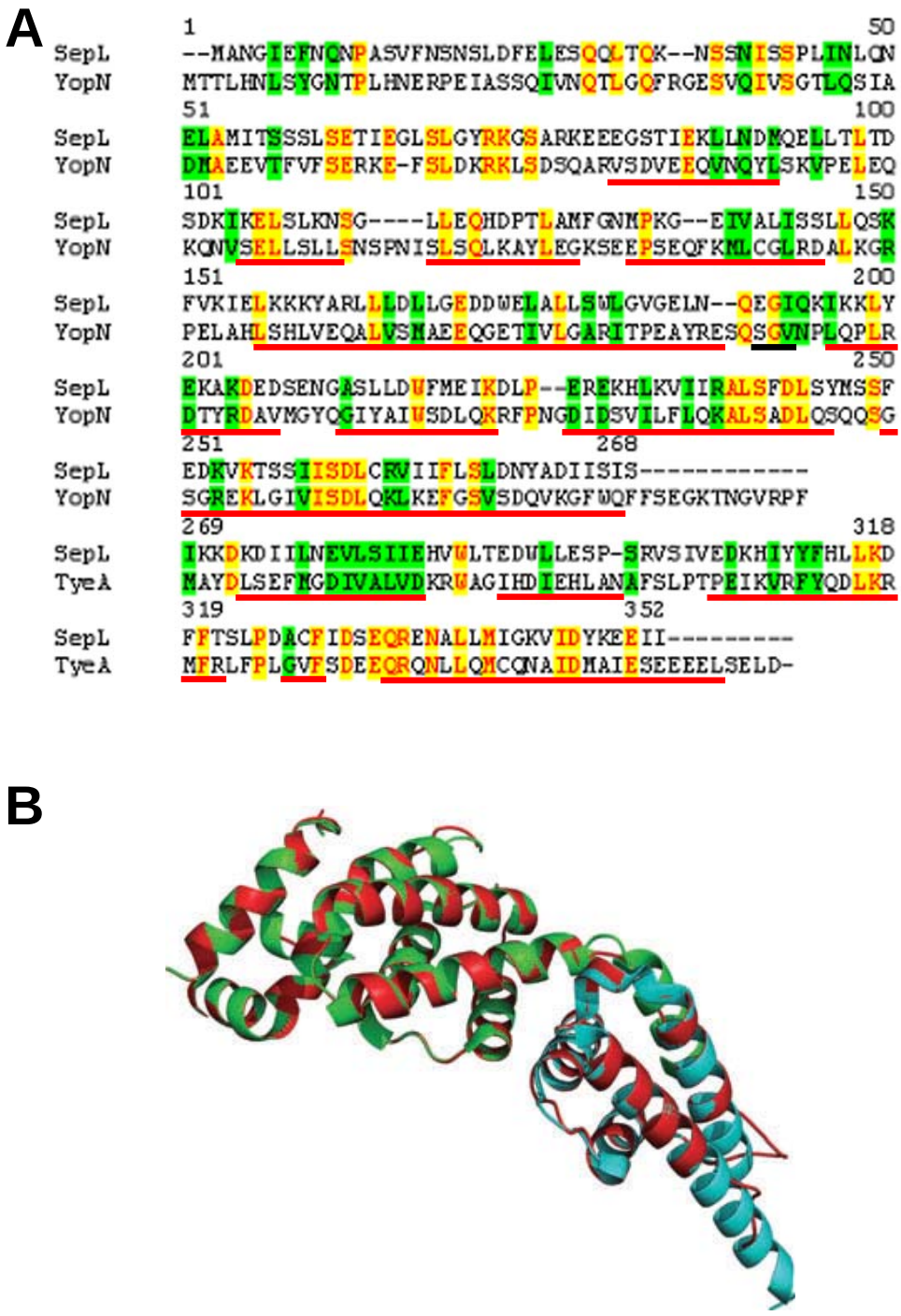


Fig. 3.12. Protein sequence and predicted structural comparisons between SepL and YopN / TyeA.
A. Amino acid alignment of SepL (gb|AAG58821) with YopN (gb|AAS58563) and TyeA (gb|AAS58564) of *Yersinia pestis*. Align X software (Invitrogen) was used to align the first 268 aa of SepL with YopN (294 aa) and the remaining 84 aa of SepL with TyeA (93 aa). Identical aa are shown by red text on a yellow background; blocks of similar amino acids are highlighted in green. (Red bars represent regions with helix structure and black bars represent regions with strand structure)
B. Structure of the YopN76-293–TyeA complex overlaid with SepL (red). YopN and TyeA are depicted in green and cyan respectively. The alignment and mapping was performed using Swissmodel using the 1XL3 file from Schubot *et al*. (2005) as a model. PyMol (http://pymol.sourceforge.net/) was used to generate the figure. (Image generated by Dr. Mads Gabrielsen)

(Pallen *et al.*, 2005a)(Fig. 3.12). TyeA is involved in the export control of specific effector proteins so it was logical to investigate C-terminus deletions of SepL to see if these can separate the known activities of SepL and this did prove to be the case. Membrane localization was studied by both imaging and biochemical analysis of SepL–GFP fusions. While some cleavage of GFP was detected from these heterologous proteins it was evident that only the full-length SepL and SepL constructs with deletions of either 11 aa or 61 aa from the C-terminus were able to localize to membrane containing fractions. The same three SepL constructs were also able to interact with SepD *in vitro*. Also, the level of SepL–GFP membrane localization was reduced in a *sepD* mutant and deletion of *sepD* resulted in an asymmetric distribution of SepL-GFP in the bacterial cell. SepD is expressed from LEE2 along with other T3S proteins. The LEE2 operon encodes several T3S basal apparatus proteins (EscC, EscJ, EscI), an effector protein (SepZ/EspZ) and a chaperone protein (CesD) besides SepD. *sepD* ORF is located between *escC* and *escJ* which both encode T3S basal protein. It is likely that the SepD is part of T3 basal structure and SepL/SepD binding is responsible for the membrane localization of SepL, possibly to the T3S apparatus, although further investigation is required to verify this. This work confirms previous research that indicated that SepL can be membrane-associated (Kresse *et al.*, 2000, Deng *et al.*, 2005), although it was reported for EPEC that SepL is only present in the cytoplasm (O'Connell *et al.*, 2004). O'Connell *et al.* also concluded that effector proteins were not hyper-secreted in a *sepL* mutant, although this does not agree with this study and other published research (Kresse *et al.*, 2000, Deng *et al.*, 2005, Deng *et al.*, 2004). In our work, the SepL–GFP fusion is present in the cytoplasm as well being localized at the bacterial membrane. The work reveals that SepL is unlikely to be a

membrane-inserted protein but is probably associated with the membrane via interaction with SepD and this localization would require the activation/maturation of the T3SS in EHEC. It is supported by the observation that the ratio of membrane localised SepL-GFP in the different genetic backgrounds were variable (Fig 3.3A). In a *sepD* deletion background, much less SepL-GFP was detected in the membrane fraction which suggested the importance of SepD in SepL localization. Ler is an activator of EHEC T3SS which initiates the expression of T3 basal apparatus proteins. Also, in my study, impaired SepL-GFP membrane localization was observed in a *ler* knockout strain. It implied that the expression or maturation of T3 basal proteins is essential for SepL membrane localization. Possibly a T3 basal apparatus related platform or binding site is required for SepL localization.

Translocon protein export was stopped by deleting the 61 aa C-terminus of SepL, even though this SepL truncate still binds SepD and localizes to the membrane as does full length SepL. As demonstrated in my study, only the full-length SepL protein was able to restore normal levels of EspADB translocator and effector protein secretion in a *sepL* mutant. However, the 11 aa deletion of SepL still functioned to some extent to export translocon proteins but had lost effector protein secretion control. Therefore the possible interaction of any of these hyper-secreted proteins with SepL was investigated. Tir was shown to interact with SepL and this interaction required the carboxy-terminal 11 aa of SepL which implied the final 11 aa of SepL were essential for controlling effector protein secretion by directly interacting with Tir. In addition, when fused to a GST tag, the C-terminal 48 aa of SepL was capable of forming a molecule which bound Tir (Fig. 3.8E).

When the structure of the YopN76-293–TyeA complex (Schubot *et al.*, 2005) is overlaid with SepL, it is evident that the final 48 aa of SepL map to the final two alpha helices of TyeA and this domain is sufficient to interact with Tir. The sequence divergence at the C-terminal 12 aa between TyeA and SepL may suggest a difference in protein recognition between the two pathogens. It has been reported that TyeA is required for *Yersinia* outer protein (Yop) secretion and YopN polarization to the bacterial membrane. However, TyeA is only required for the secretion of some Yops (YopE and YopH) but not all of them (Iriarte *et al.*, 1998). As with *Yersinia* spp., not all the effectors were regulated by the SepL/SepD switch module in EHEC. While several effector proteins are hypersecreted in a *sepL* or *sepD* mutant, such as Tir and NleA (Deng *et al.*, 2005), other research has shown that a group of effectors (EspF and EspG) are not over-secreted in EHEC by deleting *sepL* or *sepD* (O'Connell *et al.*, 2004). Therefore, in AE forming *Escherichia coli*, secreted effector proteins might be divided into two groups which are: 1.) SepL/SepD regulated (hypersecreted in a *sepL* or *sepD* mutant) and 2.) not SepL/SepD regulated effectors (secreted normally in a *sepL* or *sepD* mutant). However, among the SepL/SepD regulated effectors, Tir was the only one which was detected to bind SepL directly. There was no evidence from our work that any of the other secreted effector proteins could interact with SepL directly although they have not all been tested individually. The Far-Western analysis only indicated one binding partner, Tir. While another effector -NleA is known to be over-secreted as well in a *sepL* mutant, it did not bind to SepL *in vitro* using similar approaches that were successful with Tir. Therefore, it appears likely that the Tir–SepL interaction is critical in limiting the secretion of effector proteins in general. This finding fits well with recently published research (Thomas *et al.*, 2007)

that demonstrated that Tir is required for hyper-secretion of other effector proteins in a *sepD* mutant background.

Our previous work has demonstrated that LEE4 and LEE5 are coordinately expressed, indicating that Tir will be produced in individual bacteria while the translocon is being assembled (Roe *et al.*, 2004). Therefore, we investigated the hypothesis that the binding of Tir by SepL actually sequesters it and prevents its early release while the translocon is being assembled. The timing of release of Tir and the translocon protein EspD were investigated using a shift in culture conditions from a non-permissive to a permissive medium for T3S. Under these conditions, Tir secretion was demonstrated to be delayed in the wild type and a *sepL* deletion strain complemented by full-length SepL. However, Tir secretion occurred at the same time as EspD secretion when Δ*sepL* was complemented with SepL deleted for the C-terminal 11 aa. The data support the proposition that the timing of Tir and effector protein secretion is directly controlled by SepL binding to Tir. It also explained the effector hypersecretion phenotype of *sepL* mutants. The altered timing of EHEC T3SS probably results in an accumulation of SepL regulated effectors in the culture medium as these can be exported over a longer period by each cell. However, as the deletion of the C-terminus of SepL will have other effects on SepL function, we cannot rule out that another mechanism may be responsible for limiting effector protein export.

How Tir binding to SepL could prevent secretion of other effector proteins is not understood but it must presumably stall a series of T3S apparatus

interactions with effector proteins prior to EscN/ATPase-driven export. Another key question is how such a mechanism is then switched from translocon substrate export to effector substrate export. It has been suggested that opening a conduit to the host cell via the translocon could induce a change in local ion concentrations in particular calcium, which may change the SepL/SepD complex interaction (Deng *et al.*, 2005). My work has indicated that the SepL–Tir interaction could also be a target for the alteration of calcium concentration. Alternatively, SepL and/or SepD may have limited stability so their activity is only for a defined period. Although it has been proposed by Pallen *et al.*, 2005 that SepL, like YopN, may be secreted into bacterial supernatant to initiate effector secretion *via* T3SS (Pallen *et al.*, 2005a), no evidence was obtained to support this hypothesis in my study using 6x Histidine tagged SepL truncate. I tested whether His-tagged fusions to SepL or the SepL region homologous to YopN (the first 267 aa of SepL) are exported into the bacterial supernatant. They were not detectable in cultured media though both were expressed inside the bacterial cell (unpublished data, Dai Wang and David L. Gally) which agreed with other research (Younis *et al.*). An indication of a potential mechanism to release Tir from SepL comes from previous research that has shown a direct interaction between Tir and EscD (Pas) (Kresse *et al.*, 1998). EscD, formerly known as Pas, is an inner membrane component of the T3 basal apparatus which is required for both translocon and effector secretion (Kresse *et al.*, 1998, Ogino *et al.*, 2006). However, plasmid complementation of *escD* led to high levels of Tir secretion (Ogino *et al.*, 2006), indicating that overexpression of EscD results in loss of Tir secretion control. A number of domains have been identified in EscD, apart from two transmembrane domains, two other conserved domains have been identified potentially

providing insight into its function in the T3 basal apparatus (Kresse *et al.*, 1998, Pallen *et al.*, 2005a). Pallen *et al.* revealed a putative phospholipid-binding domain (also known as a BON domain) in the periplasmic part of EscD and a forkhead-associated (FHA) domain in its cytoplasmic part (Pallen *et al.*, 2005a). The BON domain of EscD was presumed involving in membrane binding activity (Pallen *et al.*, 2005a, Yeats and Bateman, 2003). The FHA domain was originally discovered in transcription factors and has been reported involved in protein-protein interaction and signalling events (Hofmann and Bucher, 1995). In bacterial T3SS, this FHA domain of EscD might also mediate signaling within bacterium possibly via reversible protein phosphorylation of its binding partner (Pallen *et al.*, 2002, Durocher *et al.*, 2000, Durocher and Jackson, 2002). However, it is still unknown which of EscD partner proteins might be involved in phosphorylation/dephosphorylation of serine or threonine residues. From my work, full length SepL binds directly to EscD (Fig. 3.11) and this binding requires the same final 11 aa of SepL that are required for SepL –Tir interaction. In fact, the SepL-Tir interaction can be disassociated by adding extra EscD *in vitro* (Fig. 3.11). It is also hinted at in previous research that only the cytoplasmic EscD binds to Tir, not EscD in a membrane containing fraction. This contradiction implies that EscD is able to bind to Tir, but this interaction would be interrupted by another membrane associated protein, probably SepL. Therefore, one hypothesis is that native expressed EscD (low level) would not bind to Tir until SepL/SepD complex is not membrane associated any more or degraded following sensing the outer signals, but over-expressed EscD (high level) would be able to interact with Tir by competing with SepL/SepD which might result in Tir over-secretion when bringing back a plasmid based EscD; Alternatively, there could be two

folding patterns of EscD and only the cytoplasmic version can interact with Tir but not the membrane one. Environmental signals received by T3SS could somehow alter the configuration of membrane localized EscD to a cytoplasmic version, allowing it to bind to Tir by competing with SepL. This hypothesis is supported by the size difference between cytoplasm and membrane localized EscD molecules. The latter mechanism might also be applicable to the folding of the SepL–SepD complex which is required to permit translocator and effector protein export with different binding partners at the C-terminus of SepL.

Another important factor, CesT, was previously shown to be required as a chaperone protein for efficient secretion and translocation of Tir. The CesT-Tir complex is able to interact with the ATPase, EscN, which then energizes the export of Tir (Gauthier and Finlay, 2003). CesT is known as a multi-functional protein which binds many other effector proteins, especially those known to be hypersecreted in a *sepL* mutant (Thomas *et al.*, 2005). As shown by different research groups, CesT could bind to Tir and this binding is required to stabilize Tir inside of bacterial cells for Tir export (Abe *et al.*, 1999). Further studies revealed an N-terminal chaperone binding domain in Tir (Elliott *et al.*, 1999a). My GST pull down data indicated at least two CesT binding domains in Tir including identification of a novel C-terminal binding site. However, multiple chaperone binding sites for Tir have been suggested by others (Elliott *et al.*, 1999a). It remains possible that a C-terminal domain is required for Tir secretion while an N-terminal domain is necessary for its stability. In the context of the current work, SepL and CesT were shown to bind in the same C-terminal half of Tir and our preliminary data support a hypothesis of competitive interactions between SepL and

CesT for Tir binding. Previous work in our laboratory demonstrated that LEE4 (SepL) and LEE5 (Tir) are co-ordinately expressed (Roe *et al.*, 2004). *cesT* is also located on the LEE5 operon as adjacent to *tir*. Therefore these three proteins (SepL, Tir and CesT) would be expressed simultaneously when EHEC T3SS is activated. Consequently, Tir stabilized by CesT will be present in the cell and ready to be exported while EspA filaments are assembled. However, at this time, I propose that Tir secretion is prevented by the SepD-SepL 'filter' by a Tir-SepL interaction and this interaction becomes the stalling point for effector protein secretion. Once Tir export is triggered by disassociation of this interaction, other effectors can then be exported but these also have to pass through the SepD–SepL 'filter', perhaps requiring an interaction of with CesT and/or effectors with SepD.

Overall, hierarchical secretion of T3 secreted proteins would generate a more efficient delivery system providing a selective advantage during infection. In EHEC O157, secretion of Tir and effector proteins would be restricted until the translocon is assembled so they can be secreted directly into the host cell and it would avoid unnecessary secretion. On the other hand, if Tir is secreted prior to this it may interfere with translocon assembly and therefore prolong the time scale of T3SS filament maturation and further delay injecting effector proteins into host cells. More importantly, pre-matured Tir export may bind to the bacterial surface component-intimin and therefore block the subsequent interaction of intimin with host membrane-inserted Tir. A/E *Escherichia coli* along with *Citrobacter rodentium* are unique in having SepL and SepD proteins and are the only bacterial pathogens to date known to inject their own receptor. This may not be a coincidence as tight control over the release of this receptor is required.

Chapter 4

Two secretion signal pathways:

delivery regulation of type 3 secreted proteins

4.1 Introduction

The T3SS is a complex organelle that promotes the secretion of translocon and effector proteins without disrupting the integrity of the bacterium. T3SS assembly can simplistically be described in three steps: the first step, the expression and engagement of the basal apparatus proteins (Chilcott and Hughes, 2000, Tamano *et al.*, 2000); the second step, the expression and assembly of translocon proteins on the bacterial surface surface (Menard *et al.*, 1994, Delahay *et al.*, 1999); the third step, EspADB conduit completion and the switch to effector protein translocation into the host cell (Knutton *et al.*, 1998, Ide *et al.*, 2001, Daniell *et al.*, 2001, Thomas *et al.*, 2007) .

Multiple virulence factors were found to be exported into the bacterial supernatant via the T3SS by an EHEC wild type strain (Elliott *et al.*, 2000). Moreover, other putative T3 secreted effectors, exported in a CesT-dependent manner, were discovered when either *sepL* or *sepD* were deleted (Deng *et al.*, 2004, Li *et al.*, 2006, Thomas *et al.*, 2005, Tobe *et al.*, 2006). In *sepL* or *sepD* backgrounds, Tir and Nle proteins were all identified as being over-secreted. Tir is a receptor for intimin which is crucial for bacteria-epithelial cell intimate attachment (Kenny *et al.*, 1997b, Liu *et al.*, 1999) and its secretion is required for effector hypersecretion (Thomas *et al.*, 2007). Nle proteins are less understood although some of them, such as NleA and NleB, have been shown to be translocated into host cells and important for EHEC colonization (Gruenheid *et al.*, 2004, Thanabalasuriar *et al.*, 2010, Kelly *et al.*, 2006); ongoing research is defining the functions of these additional effectors.(Tobe *et al.*, 2006) Obviously, SepL/SepD complex is controlling the secretion level of effector proteins and this control is

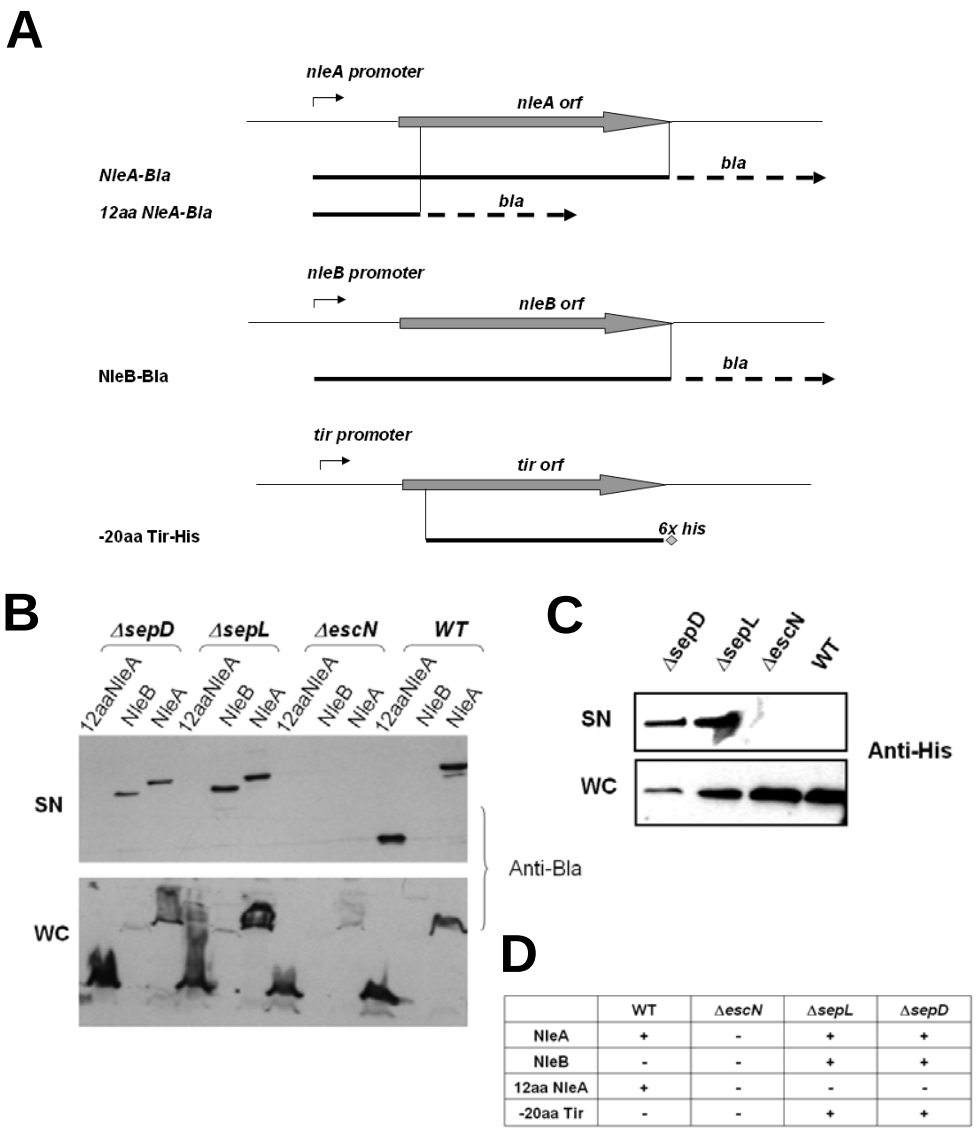
potentially critical on cells and *in vivo* due to different aspects of effectors' function (Gruenheid *et al.*, 2004, Mundy *et al.*, 2004a, Kelly *et al.*, 2006, Deng *et al.*, 2004).

T3S effectors need be targeted to the system before being secreted as they are present in the cytoplasm together with thousands of other proteins. Two secretion related domains (the N-terminal secretion signal - NSS and the chaperone binding domain - CBD) were demonstrated for YopE and therefore two independent secretion pathways have been proposed in *Yersina* spp. (Cheng *et al.*, 1997). The first (NSS) pathway was able to initiate effector protein secretion via a short peptide recognized by the needle complex which normally appears at the N-terminal of the protein and many effectors have been shown to have what may be the 'original' secretion signal (Wattiau *et al.*, 1994, Sory *et al.*, 1995, Crawford and Kaper, 2002, Lloyd *et al.*, 2002, Charpentier and Oswald, 2004). However, effectors with their NSS but lacking their chaperones were observed being secreted via T3 flagella instead of the T3SS injectisome which implied that chaperone binding delivery mediated by the interaction between chaperone and the CBD of the effector confers effector targeting/secretion specificity (Ramamurthi and Schneewind, 2003a, Lee and Galan, 2004, Badea *et al.*, 2009). In my study, to further understand the secretion controlling mechanism of the SepL-SepD protein complex, these two secretion pathways were tested in EHEC. Work in this chapter was not completed due to the time limitation of my PhD. However, it still provided some interesting observations and I propose a model which needs to be verified by future study.

4.2 Results

4.2.1 Two independent secretion pathways of EHEC T3SS

The first 15 amino acids of Tir were shown to be sufficient to signal T3 secretion and translocation into host cells without CesT (Crawford and Kaper, 2002). To my knowledge the concept of two secretion signals has not been investigated in EHEC/EPEC/CR. I wanted to test whether there are two secretion pathways for EHEC effectors and how the signals work in EHEC T3SS. Various fusions were made and tested in different genetic backgrounds to try and answer these questions. In this study, the N-terminal 12 aa section of NleA was fused to β-lactamase (*bla*) to investigate its N-terminal secretion signal while Tir without its 20aa N-terminal peptide was fused to 6x Histidine to test the CBD secretion pathway. The *bla* gene reporter system has been used by several groups to measure the delivery of T3S substrates (Charpentier and Oswald, 2004, Karavolos *et al.*, 2005). The β-lactamase protein alone is not able to be secreted without a secretion signal as its Sec-dependent N terminal secretion signal has been deleted (Karavolos *et al.*, 2005). For investigating 12aa NleA secretion, a full length NleA-Bla fusion was used as a secretion positive control while NleB-Bla was used as a secretion negative control in my experiment since it was not found exported in a previous study (Roe *et al.*, 2007). In my experiment, NleB-Bla was not expressed in an EHEC wild type strain and therefore it is not a suitable secretion negative control. All the fusions were constructed as shown in Fig. 4.1A, and transformed into EHEC wild type strain ZAP 193, an *escN* mutant, a *sepL* mutant and a *sepD* mutant separately. All the strains were cultured in a T3S permissive condition (MEM-Hepes) and protein



	WT	Δ*escN*	Δ*sepL*	Δ*sepD*
NleA	+	-	+	+
NleB	-	-	+	+
12aa NleA	+	-	-	-
-20aa Tir	-	-	+	+

Fig. 4.1. Two independent secretion pathways in EHEC
The first 12 amino acids of NleA (NSS) were fused to β-lactamase and Tir truncate missing the first 20 amino acids was fused to 6x Histidine as shown in (A). These fusions were tested in different EHEC genetic backgrounds (WT, Δ*escN*, Δ*sepL* and Δ*sepD*) (B and C). Supernatant (SN) and whole cell (WC) fractions were prepared as described in Materials and Methods. Samples were separated by 15% SDS PAGE and western blots were carried out using a β-lactamase antibody or a pentax Histidine antibody as described in Materials and Methods. 12aa NleA was exported by wild type EHEC but not by others (B). -20aa Tir secretion was detected in both Δ*sepL* and Δ*sepD* but neither EHEC wild type strain nor an *escN* mutant (C). Therefore, NSS pathway is SepL-SepD dependent but CBD pathway is inhibited by SepL-SepD complex. (D) Summary of fusion protein secretion in different genetic backgrounds.

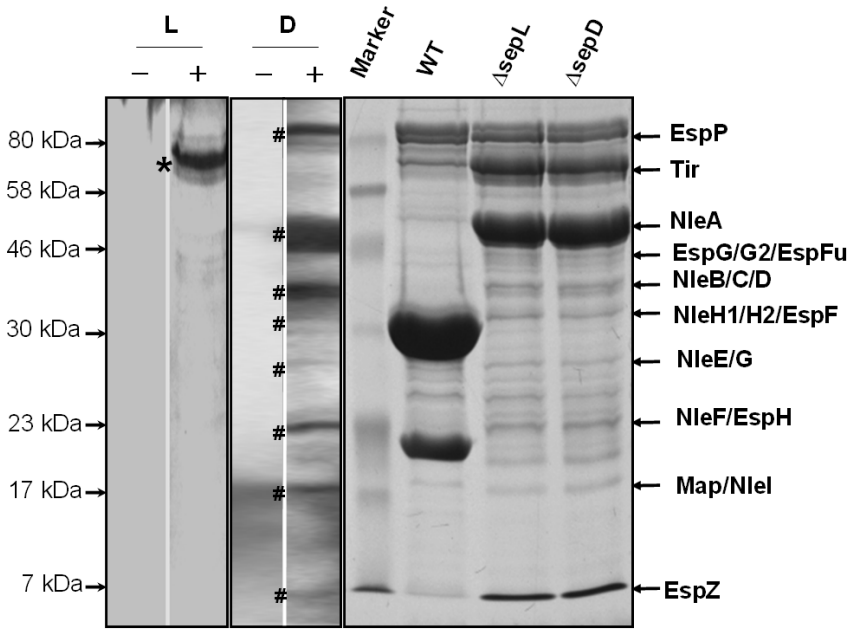
samples were prepared as described in Materials and Methods. Using a β-lactamase or a penta-Histidine antibody, it was demonstrated that the 12aa

NleA-Bla fusion was only detected in the supernatant of ZAP193 but not in the supernatant of Δ*sepL*, Δ*sepD* or Δ*escN* (Fig. 4.1B). However, -20aa Tir, which has lost its N-terminal signal, was detected in the supernatant of Δ*sepL* and Δ*sepD*, but not in the supernatant of the wild type EHEC and *escN* mutant (Fig. 4.1C). In brief, this short N-terminal secretion signal (12aa) of NleA was able to be exported via a functional EHEC T3SS but required SepL-SepD complex whereas an effector (Tir) lacking its NSS (-20aa Tir) was only exported in the absence of SepL-SepD complex via a functional EHEC T3SS. These results both imply that the SepL-SepD complex plays an important role in controlling effector secretion.

4.2.2 Interaction between SepD and other T3 components

My work in Chapter 3 on SepL function revealed that SepL bound to Tir but not to any other secreted proteins and this binding was critical for switching the T3SS from translocon secretion to effector substrate secretion in EHEC O157. As a part of the SepL/SepD complex, SepD might also interact with other T3 effectors directly which would help to explain the SepL-SepD complex requirement for the NSS secretion. The aim of the following experiments was to screen for potential binding partner of SepD.

A *sepD* mutant was cultured in a T3S permissive condition and the over-secreted protein was precipitated by adding 10% TCA into the culture supernatant. This secreted protein sample was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. This membrane was incubated with a K-12 lysate containing 6x Histidine-tagged SepD and a far-Western blot carried out using a penta-His antibody. Multiple bands were detected



Name	Estimated MW (KDa)	Apparent MW (KDa)
Tir	56.6	70
NleA (EspI)	47.6	47
EspG/EspG2	44	-
NleC	40	-
EspFu (TccP)	37.2	42
NleB	39	-
NleH1/NleH2	32.3	30
EspF	31	-
NleD	28	-
NleE	27	-
NleG	26	-
NleF	24	-
Map	23	20
NleI	20.3	18
EspZ(SepZ)	9.5	6.5

Fig. 4.2 Interaction between SepL/SepD and hypersecreted effector proteins
The supernatant samples were prepared from different strains and separated by 15% SDS PAGE (top panel right) as described in materials and methods. Far-western blot was carried out using BL21 lysate containing 6x His tagged SepD (top panel middle) or SepL (top panel left). Multiple bands were detected (labelled * for SepL detection and # for SepD detection) in this far-Western analysis; the possible interacting proteins are listed in the table.

that bound SepD and the sizes of these molecules were equivalent to most of the over-secreted effector proteins but not Tir. Tir was proposed to be the first effector protein being delivered into host cells and also showed an interaction with SepL. As a control protein, SepL was found only binding to Tir but not any other effector proteins. This far-Western result suggested that SepD interacts with other over-secreted effector proteins but not Tir directly (Fig. 4.2).

While I have shown that SepD is required for SepL membrane localization and that SepL complexed to Tir was proposed to act as a block for effector secretion, there is no further report on the function of SepD in controlling effector secretion. A key question is what does SepD do in the EHEC T3S process and why does SepD potentially bind to so many different effectors. So the potential of these SepD interactions were investigated further; the possible interaction of SepD and the NSS of NleA was tested using a GST pull-down assay. A GFP tag was fused to the C-terminal of Map and the first 12aa of NleA. The coding sequences of SepL, SepD and CesT were also inserted into pGEX 4T-2 to generate the different GST fusions. GST-tagged and GFP-tagged fusion proteins were expressed in BL21 and GST pull-down assays carried out as described in Materials and Methods. As a positive control, interactions were investigated between GST-CesT and Map-GFP. As expected, there were no interactions detected between GST-SepL/Map-GFP, GST-CesT/12aa NleA-GFP or GST-SepL/12aa NleA-GFP. Surprisingly, there was an interaction detected between GST-SepD/12aa NleA-GFP but not between GST-SepD/Map-GFP (Fig. 4.3). From the SepD far-Western result, it was known that SepD might be able to bind other over-secreted effectors but not Tir. This detection indicates that the NSS of NleA

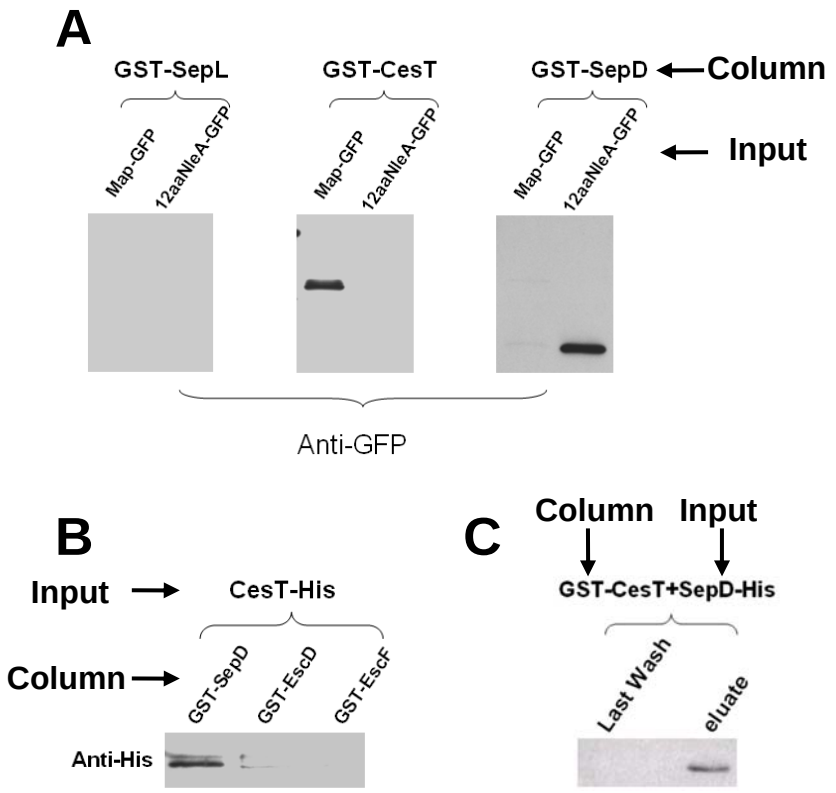


Fig. 4.3 SepD binding capacities
GST tagged SepL, SepD, EscD, EscF and CesT proteins were expressed in K12 strain-DH5α. The fusion proteins was immobilized on the G-Sephorase 4B (Amersham) columns and DH5α lysates containing different GFP (or Histidine) tagged proteins were put through these columns separately. The columns were washed 3 times with PBS following the flow through and GST tagged proteins were eluted by reduced Glutathione. Eluates were separated by 15% SDS PAGE and Western blots were carried out using a GFP (or penta-His) antibody as described in Materials and Methods. SepD was detected interacting with CesT (B and C) and the NSS of NleA (A) directly.

can bind to SepD directly. And the interaction between SepD and CesT was detected using the the same strategy (Fig. 4.3). The chaperone binding capacity of SepD implies that SepD may be involved in chaperone-mediated secretion. While this short section raises more questions than answers it does indicate further functions of the SepL/SepD complex and a model is discussed below.

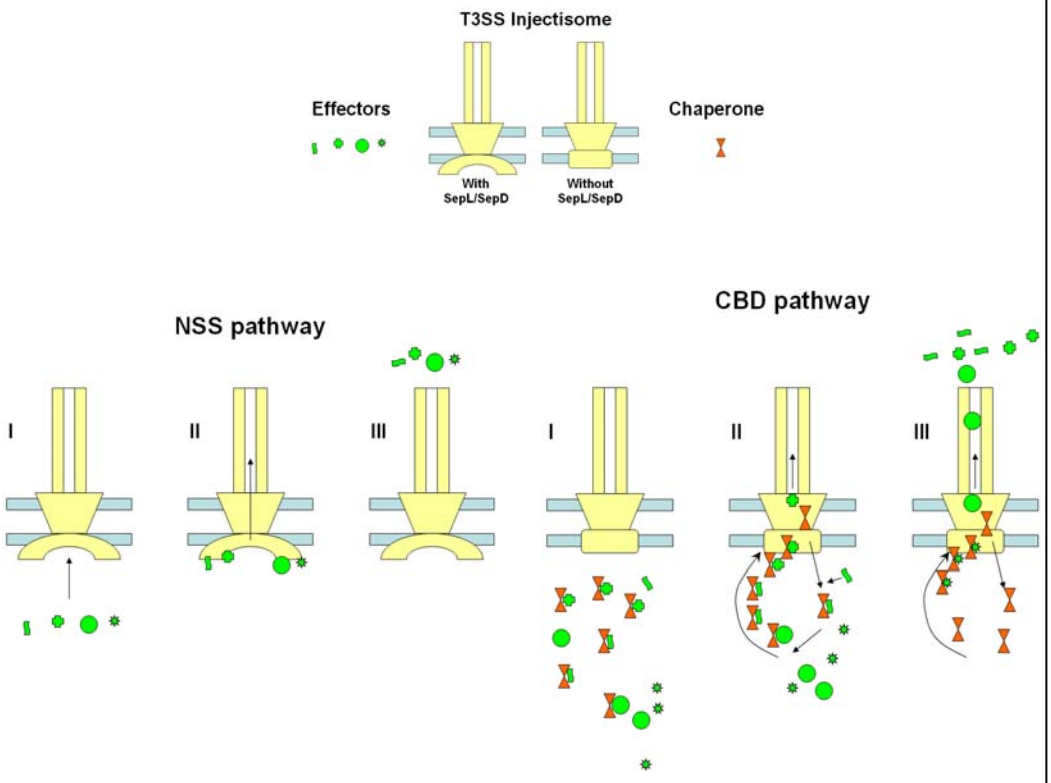
4.3 Discussion

There are many T3S effector proteins identified in EHEC/EPEC/CR (Kenny *et al.*, 1997b, McNamara and Donnenberg, 1998, Deng *et al.*, 2004, Tobe *et al.*, 2006). Unlike the structural proteins of the EHEC T3SS which are encoded by the genes on the LEE, effector proteins are encoded not only by the genes on the LEE but also on cryptic lambdoid prophages distributed throughout the EHEC genome (Deng *et al.*, 2004, Tobe *et al.*, 2006). These proteins are able to be delivered into host cells via the T3SS and are involved in modulating host cell functions. While the function of these effector proteins is the subject of extensive research,, it is also important to understand the mechanism and regulation of effector delivery. As shown in EHEC/EPEC/CR, there is an ordered cascade of protein secretion (Deng *et al.*, 2005) and Tir is proposed to be the first effector protein to be translocated into host cells (Mills *et al.*, 2008, Thomas *et al.*, 2007), consistent with my work in Chapter 3.

Interestingly, the T3 secretion of effectors is enhanced when *sepL* or *sepD* are deleted (Deng *et al.*, 2005). This over-secretion phenotype led initially to more than 8 novel effector proteins being identified from a *sepL* mutant(Deng *et al.*, 2004) with further work extending this to 39 additional proteins (Tobe *et al.*, 2006) and some of them were not even detected in the supernatant of wild type EHEC (Roe *et al.*, 2007). In order to understand the regulation of effector secretion, two secretion pathways were tested in EHEC and *sepL*/*sepD* mutants. It was confirmed that there were two independent secretion pathways for T3 effectors in EHEC according to my observations. Further examination showed that the NSS of NleA cannot be secreted via

Box 1: two independent secretion pathways in EHEC

Although some information about two secretion pathways in EHEC were discovered by my work, further work is required to define how these secretion signals work for the export of the effector proteins. Here, I propose models of these two secretion pathways in EHEC based on our current research and previous reports. As shown above, the NSS of NleA is able to initiate the effector export in a wild type EHEC which required the SepL/SepD complex and the CBD region is responsible for the effector over-secretion in *sepL*/*sepD* mutants. The model for the NSS pathway therefore includes the SepL-SepD complex engaging at the T3 basal structure: I, the SepL/SepD complex is localized to the T3 basal apparatus; II. Effectors are self-targeted to the T3SS by the NSS/SepD interaction; III. Effectors are exported by the T3SS. However, EHEC effectors actually could not be secreted in this manner due to the dominant CBD sequence which controls the injection, potentially in a more specific manner. With their CBD regions, effector secretion was mediated via their chaperones in T3SS (Thomas *et al.*, 2007). The model for CBD –dependent secretion is more likely to be the main pathway used during EHEC infection. In this model, SepL-SepD are no longer complexed at the T3SS which could promote the CBD secretion pathway: I. SepL-SepD complex disassociates from the T3SS and effectors are chaperoned by CesT; II. CesT delivers effectors to the ATPase according to binding affinities; III. Ordered export of effectors.



EHEC T3SS in the absence of theSepL-SepD complex. However Tir without the NSS and therefore only with the CBD signal is behaving in the opposite way, being inhibited by the presence of the SepL-SepD complex. This evidence indicates that the SepL-SepD complex may be important for

switching between these two independent secretion pathways (proposed models see Box 1).

To achieve regulation of effector secretion, SepL-SepD complex needs a mechanism to block the CBD signal and facilitate the use of the NSS. My study (Far Western analysis) demonstrates that SepD acts as a binding partner for many EHEC T3 effectors and the NSS of NleA was shown to interact with SepD directly using a GST pull down experiment. SepD also binds to a multi-functional chaperone protein CesT according to my study. Taken together, I suggest that SepD in the SepL-SepD complex works as a potential targeting site for this 'original' N-terminal secretion signal. However, I propose that the CBD pathway is a more efficient way for protein translocation to occur after the NSS targeting and so the CBD has surpassed the NSS as a secretion (as opposed to targeting) signal. The CBD secretion pathway might be inhibited by the SepL-SepD complex via the interaction between SepD and CesT in EHEC.

CesT is a multifunctional chaperone which is required for stabilizing effectors and the over-secretion phenotype of *sepL*/*sepD* mutant (Thomas *et al.*, 2005, Thomas *et al.*, 2007). Previous research has already showed that CesT interacts with multiple effector proteins using a CesT affinity column in EPEC (Thomas *et al.*, 2005). An escort mechanism for cycling of export chaperones during T3 flagellum assembly was reported previously (Evans *et al.*, 2006). For chaperone cycling, normally there is a docking area for the accumulation of secreted proteins and chaperones. It was suggested that C ring protein acted as a docking sites for T3 effectors and chaperones (Gonzalez-Pedrajo *et al.*, 2006, Spaeth *et al.*, 2009). But a C-ring component

has not yet been identified in EHEC/EPEC/CR. Although SepD doesn't share a significant sequence similarity with other C ring components according to a multiple sequence alignment (Pallen *et al.*, 2005a), it remains a possibility that SepL-SepD complex interacting with a C-ring component. An escort mechanism for cycling of export chaperones in EHEC was supported by SepD binding to CesT and the NSS of NleA.

When the assembly of T3SS injectisome is completed in EHEC, effectors need to be targeted to T3SS for secretion/translocation. They could be targeted to the T3 basal apparatus via the interaction between SepD and the NSS according to my data, or could be carried to ATPase (EscN) by a chaperone protein (Gauthier and Finlay, 2003). As proposed in chapter 3, SepL may release Tir to initiate effector secretion after EspA filament completed. Therefore, the NSS secretion pathway which required SepL-SepD complex cannot be mainly responsible for effector secretion as SepL-SepD complex would sequester Tir secretion in EHEC. However, the NSS signal could act as a perfect T3SS targeting signal facilitating CesT mediated effector secretion which didn't require SepL-SepD complex. Logically, CesT mediated secretion would be the major approach used injecting virulence factor during the infection.

Although some evidence was obtained in my research to support two secretion signal pathways (1. The NSS targeting and 2. CBD/chaperone mediated export) facilitating effector secretion regulation in EHEC O157, it remains unclear whether this mechanism is applicable for all other T3SS. More and more published work suggested that the NSS is sufficient for effector targeting / secretion via T3SS and CBD/chaperone interaction

confer T3SS specificity (Cheng *et al.*, 1997, Cheng and Schneewind, 2000a, Lloyd *et al.*, 2001a, Lloyd *et al.*, 2001b, Crawford and Kaper, 2002, Birtalan *et al.*, 2002, Lloyd *et al.*, 2002, Ramamurthi and Schneewind, 2003b, Ramamurthi and Schneewind, 2003a, Sorg *et al.*, 2005, Higashide and Zhou, 2006, Rodgers *et al.*, 2008, Badea *et al.*, 2009). Although as mentioned in Chapter 1, the phenotype discrepancy suggested SepL and its homologues (YopN-TyeA, SsaL, MxiC, PopN…) were behaving in slightly different ways, they were all responsible for translocon/effector substrate switching (Yang *et al.*, 2007, Yu *et al.*, 2010, Coombes *et al.*, 2004, Torruellas *et al.*, 2005, Ferracci *et al.*, 2005, Schubot *et al.*, 2005, Younis *et al.*, Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Pallen *et al.*, 2005b, Deng *et al.*, 2005, Pallen *et al.*, 2005a). Previous studies in *Salmonella* suggested that a protein complex (SsaM-SsaL-SpiC) controlling effector secretion could be disassociated by pH change (Coombes *et al.*, 2004, Yu *et al.*, 2010). Moreover, recent studies identified homologues of this protein complex in other T3SS which suggested functional similarity among SsaM-SsaL-SpiC (*Salmonella* SPI-2), SycN-YopN-TyeA-YscB (*Yersinia*), and CesL(Mpc)-SepL-SepD (EHEC/EPEC/CR) (Pallen *et al.*, 2005a, Younis *et al.*, 2010). However, the SpiC/SepD/YscB family proteins have shown phenotypic diversity. YscB is known as a chaperone protein for YopN and SpiC is a secreted virulence protein (Day and Plano, 1998, Jackson *et al.*, 1998, Cheng *et al.*, 2001, Uchiya *et al.*, 1999, Freeman *et al.*, 2002, Yu *et al.*, 2002). Furthermore, according to previous and my research, SepD was only known as a membrane-associated protein involved in T3 secretion switching and SepL localisation (O'Connell *et al.*, 2004, Deng *et al.*, 2005, Wang *et al.*, 2008). It remains possible that the SepD/SpiC family of proteins act as a T3SS docking site for recruiting and recycling of effector/chaperone,

therefore my proposal suggests an intriguing area for future work on how T3SS is controlled by environmental signals.

4.4 Future work

4.4.1 The NSS signals of EHEC secreted proteins: mRNA or amino acids?

From previous reports and our own studies, it is known that the NSS is located in the N terminal region of secreted proteins and capable directing effector protein to host cells (Anderson and Schneewind, 1997, Cheng *et al.*, 1997, Lloyd *et al.*, 2001b, Ramamurthi and Schneewind, 2002). But it is still unclear whether it is an mRNA signal or amino acid signal for EHEC secreted proteins. So the next aim is to define the nature of the EHEC NSS. The NSS of *Yersinia* secreted proteins was studied by Wolf-Watz and Schneewind's groups (Anderson and Schneewind, 1997, Cheng *et al.*, 1997, Lloyd *et al.*, 2001b, Ramamurthi and Schneewind, 2002). It was reported that YopE had two distinct secretion signals and the NSS signal is able to direct YopE to the T3 basal proteins (Cheng *et al.*, 1997). But it is still debatable whether the NSS is an mRNA or a peptide signal. Originally, Wolf-Watz and his colleagues' work suggested that YopE is targeted for T3S by N-terminal, not mRNA, signal (Schesser *et al.*, 1996). However, a year later, Schneewind' group published their research which revealed a signal led to T3S of Yop proteins is contained in their 5' messenger RNA rather than the peptide sequence (Anderson and Schneewind, 1997). Similar studies were carried out in *Salmonella enterica* by Galán and his colleagues and their results also suggested possible secretion signals (Lee and Galan, 2004). So far no study has reported investigating the secretion signals of EHEC secreted proteins in details. Our work showed that there were two

secretion pathways of EHEC secreted proteins and the NSS pathway requires the presence of SepL/SepD in EHEC. Moreover, our binding assay showed that the NSS of NleA was able to bind to SepD directly. Although it implied that the NSS of NleA was more likely to be a peptide signal rather than an mRNA signal, it is still possible that the NSS of NleA is encoded by an mRNA sequence. So, in the future, the frameshift and synonymous changes would be introduced into the NSS of EHEC secreted protein and the secretion abilities of those mutants would be tested in EHEC.

4.4.2 SepD interactions

My far-Western result suggested the possible interactions between SepD and many secreted proteins. The NSS of NleA was also shown to bind to SepD directly. Logically, the future work should be focused on identifying and confirming the potential SepD binding partners. The N-terminal secretion signals of various effector proteins would be tested for SepD direct binding capacity. As discussed above, SepL-SepD was proposed to interact with a C ring component of EHEC T3SS which might be SepQ (Pallen *et al.*, 2005a) and serving as a docking site for secreted proteins and chaperones. This hypothesis would be further tested using GST pull-down or far-Western approach. Once SepL-SepD binding partners are identified, the domain within SepD required for these interaction could be mapped using truncated SepD proteins.

Chapter 5

Materials and Methods

5.1 Bacterial strains, plasmids, oligonucleotides, media and antibodies

The bacterial strains, media, antibodies and plasmids used in the study are described in Tables 5.1 & 5.2. Table 5.3 lists the oligonucleotide primers used. MEM-HEPES is minimal essential medium with 25 mM HEPES buffer (Sigma), containing additional glucose to a final concentration of 0.2%. Luria-Bertani (LB) broth was also used (Oxoid). Antibiotics were included when required at the following concentrations: chloramphenicol (cam) 12.5 μg ml^{-1}, kanamycin (kan) 25 μg ml^{-1}, ampicillin (Amp) 50 μg ml^{-1}.

5.2 Preparation of secreted proteins and bacterial fractions for protein analyses

Bacteria were cultured in 50 ml MEM HEPES at 37°C (200 rpm) to an OD_{600} of 0.8 unless specifically stated. The bacterial cells were pelleted by centrifugation at 4,000 × g for 20 min, and supernatants were passed through filters (0.45 μm). Proteins were precipitated overnight with 10% TCA, and separated by centrifugation at 4,000 × g for 30 mins (4°C), the proteins were resuspended in 150 μl 1.5 M Tris (pH 8.8). The bacterial pellet was initially suspended in 150 μl of sonication buffer (10 mM Tris-HCl [pH 7.5], 0.5 mM PMSF and 0.5 μg/ml aprotinin) and sonicated (3 × 10 seconds) on ice. Cell envelopes and unbroken bacteria were removed by two rounds of centrifugation (5,000 × g for 10 mins at 4°C). The supernatant (whole cell fraction) was removed and the membranes pelleted by ultra-centrifugation of the samples for 1 hr at 50,000 × g, at 4°C. The supernatant containing cytoplasmic proteins was collected. The membrane preparation was washed twice with sonication buffer and re-suspended in 150 μl SDS sample buffer

(63 mM Tris HCl [pH 6.8], 10% Glycerol, 2% SDS and 0.0025% Bromophenol Blue). Proteins were separated by SDS-PAGE using standard methods and Western blotting performed as described previously (Sambrook *et al.*, 1989) using the relevant antibodies listed in Table 5.2. Tir and EspD secretion levels were measured following enhanced chemi-luminescence (ECL)-detection from Western blots using Multi-analyst (Bio-Rad) software.

5.3 DNA amplification and manipulation

PCR amplification was performed as described previously (Sambrook *et al.*, 1989). Plasmid preparation and purification of PCR product according to manufacturer's instructions (QIAprep spin miniprep kit, QIAquick Gel extraction kit, QIAquick PCR purification kit) (Appendix 1)

5.4 Construction of various reporter tagged fusions

For SepL/SepD co-localization study, a fusion construct to RFP (Sorensen *et al.*, 2003) was made in pDW16. The *pTac* promoter was amplified from plasmid pGEX4T2 (Table 5.1) and cloned in front of *rfp* using primers ptac5' and ptac3' to create pDW17. The *sepD* gene was then amplified from EHEC ZAP193 using primers sepD 5'r and sepD 3'r and cloned between the *pTac* promoter and *rfp*. Full length SepL and different carboxyl-terminal truncates of SepL (Fig. 3.1A) were fused to GFP in pAJR70 (Roe *et al.*, 2003b) using the primers described in Table 5.3. A 6 × His-tag was introduced at the carboxy end of SepL and three of the truncated SepL proteins (267aa, 290aa and 340aa) by PCR before cloning into pACYC184 (Table 5.1). Different effectors were fused to a β-lactamase or 6 × His-tag

using the primers described in Table 5.3. All constructs were confirmed by sequencing and listed in Table 5.1.

5.5 Northern Analyses

Bacteria were cultured in the relevant media and harvested at OD_{600} = 0.8 in MEM HEPES and OD_{600} = 1.5 in LB due to the difference of growth rates. Total RNA was extracted using a 'ChargeSwitch Total RNA Cell Kit' (Invitrogen) as per the manufacturer's instructions (Appendix 1). Northern blotting was performed according to the NorthernMax® Kit Instruction Manual (Ambion) (Appendix 1). RNA was quantified using an Agilent 2100 Bioanalyzer. Samples were hybridized with single-stranded DNA probes specific for either *sepL* or *gfp*. The primers used to generate these probes are given in Table 5.3: for *sepL* the primers were sepL3-5' & sepL4-3'; for *egfp* the primers were egfp5'P & egfp3'P. The probes were labeled with [^{32}P]dCTP by incubating the mixture of denatured DNA probe (25-50 ng) and [^{32}P]dCTP (50 μCi) at 37 for 15 mins using Ready-To-Go DNA labelling beads (Amersham-Pharmacia Biotech). Following hybridization and washing steps, the ^{32}P signal was detected with a PhosphorImager (Bio-Rad GS-525), which allowed quantification using Multi-Analyst software (Bio-Rad).

5.6 Construction of GST and 6 x His-tagged proteins and binding assays

For the GST-SepL construct, *sepL* was amplified from EHEC O157 ZAP193 by PCR using the primers sepL 5'g and sepL 3'g. The resulting PCR product was digested with BamHI and SmaI, and cloned into the BamHI and SmaI

sites of pGEX-4T-2. This creates a GST-SepL hybrid protein fusion (in pDW9) used in GST pull-down assays. A similar strategy was used to clone the 48aa carboxyl terminus of SepL, SepD and CesT using the primers described in Table 5.3. For His-tagged proteins, *tir* and *sepD* open reading frames were amplified by the primers listed in Table 5.3, digested with *Xba*I and *Xho*I, and cloned into the *Xba*I and *Xho*I sites of pET21d. For SepL and Tir domain analyses, the amplified fragments were cloned into pET28a via *Nde*I and *Xho*I to create N-terminal 6 × His-tags. All constructs were expressed in *Escherichia coli* BL21 (Table 5.2) following IPTG induction (0.1mM). The GST fusions were expressed in AAEC 185 (an *Escherichia coli* K-12 derivative, Table 5.2) and the His-tag fusions in *Escherichia coli* BL21, both following IPTG (0.1mM) induction in LB at OD600 = 0.5. For protein preparations, the bacteria were harvested at 4,000 × g (4°C) for 30 mins 2 hours post IPTG inoculation. The bacterial pellet was suspended in Phosphate buffered saline (PBS) and sonicated. The supernatant was collected by centrifugation at 12,000 × g for 10 mins at 4°C. For the GST fusions, the supernatant was mixed with PBS balanced Glutathione-Sepharose 4B beads for 30 mins at room temperature. The beads were separated by centrifugation at 500 × g for 5 mins. An aliquot of the supernatant was saved for analysis and rest of the supernatant discarded. The beads were washed three times using 10 volumes of PBS and separation by centrifugation at 500 × g for 5 mins. The beads were mixed gently in the same volume of Glutathione Elution Buffer (0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl, pH 8.0) and incubated at room temperature for 10 mins. Supernatant was collected by centrifugation at 500 × g for 5 mins. The elution and centrifugation step was repeated and the two eluates pooled. Equal volumes of washes and eluates were loaded onto the

protein gels. To check initial loading of columns, some eluates were stained with colloidal blue and/or Western blotted to confirm the presence of the expected GST fusion protein and His or GFP-tagged binding partners.

For His-tagged proteins, these were expressed and purified as above except a Ni-NTA (Qiagen) column was used and elution was with the supplied Qiagen buffer. For the *in vitro* binding assays, these were carried out on either glutathione or Ni-NTA columns on which the bait protein was first retained and then the protein being investigated was run through the column in a lysate prepared as above. Following washes, elution was carried out as described above. Eluted samples were analysed by SDS-PAGE followed by colloidal blue staining and Western Blotting which were carried out essentially as described previously (Roe *et al.*, 2003b, Karavolos *et al.*, 2005) using the relevant antibodies listed in Table 5.2.

5.7 Construction of sepL and sepD mutants

The experiments were carried out essentially as described previously (Roe *et al.*, 2003b, Emmerson *et al.*, 2006) using allelic exchange methodology. Exchanging plasmid was transformed into appropriate strain background. 4-8 single colonies were inoculated into 100 ml LB (Cam) medium and this culture was grown at 42°C (200 rpm) for 8-16 hrs. This step was repeated 3 times by diluting (1:1000) into 100 ml fresh medium. After 4 rounds at 42°C, the culture was diluted into 100 ml LB medium with appropriate antibiotic (Kanamycin for *sacB-kana*^{R} cassette insertion, non-antibiotic added for knocking *sacB-kana*^{R} cassette out) and grown at 28°C (200 rpm) for 8-16 hrs. This step was repeated 3 times by diluting (1:1000) into 100 ml fresh

medium. After the final round of 28°C culturing, it was diluted to 10^{-6}-10^{-7} and put on LBK (for *sacB-kana*^{R} insertion) or LB (For *sacB-kana*^{R} knocking out) plate at 28°C. After 16-20 hours, single colonies should be visible and ready to be tested for antibiotic resistance and colony PCR screening. The respective primer sets used to amplify the *sepL*, *sepD* are described in Table 5.3, the *ler* deletion was published previously (Low AS, 2006). To generate plasmids for the *sepL* frameshift mutation, a fragment containing the *sepL* gene and flanking regions (Upstream 1kb+ downstream 1kb) was amplified from ZAP193 by PCR using sepL 5'allf and sepL 3'allf. It was digested with *Kpn* I and *Xba* I, and cloned into the *Kpn* I and *Xba* I sites of pIB307. Following the methodology in the Stratagene "Site-Directed Mutagensis" kit (Appendix 1), a single base pair was inserted at 345bp of sepL to generate a plasmid (pDW52) for allelic exchange. Final plasmid constructs (Table 5.1) were sequenced prior to the deletion exchange and each deletion confirmed by PCR analysis. The *sepL* and *sepD* mutants could be functionally complemented by pDW24 (SepL), pDW48 (SepL-His) pDW6 (SepL-GFP) and pDW20 (SepD-GFP) (Table 5.1) to restore translocon (EspD) secretion as determined by Western blotting (Fig. 4A, C and D).

5.8 Far-Western Analysis

Supernatant proteins were prepared from ZAP1143 (Δ*sepL*) or ZAP1144 (Δ*sepD*) as described in the relevant section above. The secreted proteins were separated by SDS-PAGE using standard methods and then transferred to a Nitrocellulose membrane (150 volt for 90 mins) (Sambrook *et al.*, 1989). The membrane was first blocked with 8% milk PBS overnight at 4°C and washed 3 times with PBS-Tween (0.5% v/v) before being incubated

overnight with saturated SepL-His or SepD-His in an *Escherichia coli* BL21 lysate at 4°C. After the incubation, the nitrocellulose membrane was washed 3 times with PBS-Tween and the standard Western procedure for detecting the 6 x His-tags was carried out (Sambrook *et al.*, 1989).

5.9 RNA folding prediction

The RNA secondary structures for different *sepL* fusions were predicted using RNADraw software, a program for RNA secondary structure calculation and analysis (Matzura and Wennborg, 1996). RNADRAW predicts RNA structures by identifying suboptimal structures using the free energy optimization methodology at a default temperature of 37°C. In this study, different length sepL coding regions with its 5' UTR fused in frame to the full length *gfp* gene were used for RNA structure prediction.

5.10 EspA filament staining

Fixed bacteria were dried on slides and incubated with a 1:100 dilution of the anti-EspA serum (Table 5.2) (PBS/0.1% BSA) for 60-90 mins at room temperature. After 3 × washes (PBS/0.1% BSA), the bacterial samples were incubated with either FITC-conjugated (Green) or TRITC-conjugated (Red) goat anti-rabbit antibody (Dako, Table 5.2) (1:500, PBS/0.1% BSA) for 30-60 mins and the slide was washed three times with PBS/0.1% BSA. The slide was then examined by fluorescence microscopy and the images were visualised and captured using Leica software as described below in section 5.12.

5.11 EBL cell binding assay

Embryonic bovine lung (EBL) cells (German collection of cell cultures, No. ACC 192) were plated at a density of 1×10^5 in eight-chamber microscope slides (Becton and Dickinson). Cells were incubated at 37°C in 5% CO_2 for 24 hrs before bacterial co-culture. EHEC O157 strains were transformed with the appropriate GFP/RFP reporter plasmid (Table 5.1) using standard protocols. The resultant transformants were cultured overnight in LB medium with appropriate antibiotic at 37°C. For the binding assays, the bacteria were then inoculated into M9 cam medium (start OD_{600}=0.05) until an OD_{600} = 0.6. Culture growth medium (MEM-Hepes) was removed from the chambers and the bacteria were added to each well of the multi-chamber slide (150 µl per chamber). The cells were then incubated at 37°C in 5% CO_2 for 1 hr. The medium was then removed and replaced with fresh M9 medium (Cam), removing any unattached bacteria. Cells were incubated for a further 1-3 hrs as required and then fixed with 200 µl of 4% paraformaldehyde(in PBS). In order to visualize EspA filaments on the surface of bacteria, EspA immuno-staining was carried out essentially as described above in section 5.10.

5.12 Fluorescence imaging

Fluorescence imaging was carried out using a Leica DM LB2 microscope and a 100 × objective lens. Narrow bandwidth filters to excite and detect GFP/FITC were used (41017 Endow GFP, CHROMA). Images were captured using a Hamamatsu ORCA-ER black and white CCD digital camera. Images were analysed using OpenLab software (Improvision). To measure levels of fluorescence in individual cells, transects were marked on

bacteria and the fluorescence levels determined using QFluor software (Leica).

Table 5.1 Plasmids used in the study (plasmids underlined were made by Dai Wang in this study)

Plasmid	Description
pACYC184	Low copy number cloning vector from New England Biolabs
pGEX-4T-2	Plasmid contained GST gene fusion system from Amersham Biosciences
pET21d	Plasmid contained 6 x His gene fusion system from Novagen
pET28a	Plasmid contained 6 x His gene fusion system from Novagen
pTir-His	pET21d digested with *Xba*I/*Xho*I; fragment contained full length *tir* gene amplified from ZAP193 and inserted
p100Tir-His	pET21d digested with *Xba*I/*Xho*I; fragment contained the first 100aa *tir* gene amplified from ZAP193 and inserted
p200Tir-His	pET21d digested with *Xba*I/*Xho*I; fragment contained the first 200aa *tir* gene amplified from ZAP193 and inserted
p-200Tir-His	pET28a digested with *Nde*I/*Xho*I; fragment contained the 201-558aa *tir* gene amplified from ZAP193 and inserted
p-382Tir-His	pET28a digested with *Nde*I/*Xho*I; fragment contained the 382-558aa *tir* gene amplified from ZAP193 and inserted
pDG028	Low copy number vector containing *sacB-kana*^{R} cassette, laboratory stock.
pIB307	pMAK705 based vector for allelic exchange; temperature sensitive replicon (Blomfield *et al.*, 1991)
pAJR70	pACYC184 digested with *BamH*I; *gfp* gene cloned *BamHl/Bgl*II (Roe *et al.*, 2003b)
pAJR104	pACYC184 containing *bla* gene (Karavolos *et al.*, 2005)
pAJR145	pACYC *rpsM*::*gfp* transcriptional fusion (Roe *et al.*, 2004)
pDW5	pAJR70 digested with *BamHl/Kpn*I; *map* with its own promoter amplified from ZAP193, cloned in frame 5' to *gfp*
pDW6	pAJR70 digested with *BamHl/Kpn*I; *sepL* with its own promoter amplified from ZAP193, cloned in frame 5' to *gfp*
pDW7	pIB307 digested with *Xba*I/*Hind*III; fragment contained 1165 bp *sepL* downstream sequence amplified from ZAP193 and inserted
pDW8	pDW7 digested with *Kpn*I/*BamH*I; fragment contained 985 bp *sepL* upstream sequence amplified from ZAP193 and inserted
pDW9	pGEX-4T-2 digested with *BamHl/Sma*I; fragment contained full length *sepL* gene amplified from ZAP193 and inserted
pDW10	pIB307 cut with *Kpn*I/*BamH*I; fragment contained 985 bp *sepL* upstream sequence amplified from ZAP193 and inserted
pDW11	pDW8 digested with *BamH*I; *sacB-kana*^{R} cassette inserted
pDW14	pDW8 digested with *Bam*HI; fragment contained *sepL-gfp* sequence amplified from pDW6 and inserted
pDW15	pGEX-4T-2 digested with *BamHl/Sma*I; fragment contained full length *sepD* gene amplified from ZAP193 and inserted

pDW16	pACYC184 containing *rfp* gene (Roe *et al.*, 2007)
pDW17	pDW16: Artificial promoter *ptac* was inserted in front of *rfp* gene
pDW18	pET21d digested with *Xba*I/*Xho*I; fragment contained full length *sepD* gene amplified from ZAP193 and inserted
pDW20	pAJR70 digested with *BamHI*/*Kpn*I; fragment contained full length *sepD* gene amplified from ZAP193, cloned in frame 5' to *egfp*
pDW21	pDW307 digested with *BamH*I/*Sac*I; fragment contained 746 bp *sepD* upstream sequence amplified from ZAP193 and inserted
pDW22	pDW21 digested with *BamHI*/*Ase*I; fragment contained 805 bp *sepD* downstream sequence amplified from ZAP193 and inserted
PDW23	pDW22 digested with *BamH*I; *sacB-kana*^{R} cassette inserted
pDW24	pACYC184 digested with *BamH*I; Full length *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW25	pDW17: *sepD* gene and its Shine-Dalgarno sequence was amplified from ZAP193 and fused to *rfp* gene in frame driven by *ptac* promoter
pDW26	pACYC184 digested with *BamHI*/*Kpn*I; 1-51bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW27	pACYC184 digested with *BamHI*/*Kpn*I; 1-210bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW28	pACYC184 digested with *BamHI*/*Kpn*I; 1-573bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW29	pACYC184 digested with *BamHI*/*Kpn*I; 1-870bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW30	pACYC184 digested with *BamHI*/*Kpn*I; 1-1020bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW40	pGEX-4T-2 digested with *BamHI*/*Sma*I; fragment contained full length *cesT* gene amplified from ZAP193 and inserted
pDW42	pET21d digested with *Xba*I/*Xho*I; fragment contained full length *sepL* gene amplified from ZAP193 and inserted
pDW45	pACYC184 digested with *BamH*I; 1-801bp *sepL* gene with its own promoter amplified from ZAP193 (6X His-tag was introduced into C-terminal by primer) and inserted
pDW46	pACYC184 digested with *BamH*I; 1-870bp *sepL* gene with its own promoter amplified from ZAP193 (6X His-tag was introduced into C-terminal by primer) and inserted
pDW47	pACYC184 digested with *BamH*I; 1-1020bp *sepL* gene with its own promoter amplified from ZAP193 (6X His-tag was introduced into C-terminal by primer) and inserted
pDW48	pACYC184 digested with *BamH*I; Full length *sepL* gene with its own promoter amplified from ZAP193 (6X His-tag was introduced into C-terminal by primer) and inserted
pDW50	pGEX-4T-2 digested with *BamHI*/*Sma*I; fragment contained caboxy-terminal 48aa residue of *sepL* gene amplified from ZAP193 and inserted

pDW51	pIB307 digested with *Kpn*I/*Xba*I; fragment contained *sepL* gene and flanking regions (Upstream 1kb+ downstream 1kb) amplified from ZAP193 and inserted
pDW52	pDW51 was site-direct mutated by inserting a base pair at 345bp of *sepL* ORF
pDW53	pIB307 digested with *BamH*I/*Ase*I; fragment contained *csrA* downstream sequence amplified from ZAP193 and inserted
pDW54	pDW53 digested with *BamH*I/*Kpn*I; fragment contained *csrA* upstream sequence amplified from ZAP193 and inserted
pDW55	pDW54 digested with *BamH*I, fragment contained *sacB-kana*^{R} cassette inserted
pDW61	pAJR70 digested with *BamH*I/*Kpn*I; 1–81 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW62	pAJR70 digested with *BamH*I/*Kpn*I; 1–84 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW63	pWSK29 digested with *Bam*HI/*EcoR*I; *hfq* gene amplified from ZAP193 and inserted
pDW66	pWSK29 digested with *Bam*HI/*EcoR*I; *csrA* gene amplified from ZAP193 and inserted
pDW64	pACYC177: *sepL-gfp* fragment restricted from pDW6 and inserted
pDW65	pACYC177: 51bp *sepL-gfp* fragment restricted from pDW26 and inserted
pDWNleA-His	pET21d digested with *Xba*I/*Xho*I; fragment contained full length *nleA* gene amplified from ZAP193 and inserted
pDWHis-NleA	pET28a digested with *Nde*I/*Xho*I; fragment contained full length *nleA* gene amplified from ZAP193 and inserted
pTD-8AT	pAJR104 digested with *BamH*I/*Kpn*I; full length *nleA* gene with its own promoter amplified from ZAP193 and inserted (Roe *et al.*, 2007)
pTD-9BT	pAJR104 digested with *BamH*I/*Kpn*I; *nleB* gene with its own promoter amplified from ZAP193 and inserted (Roe *et al.*, 2007)
pTD-1A	pAJR70 digested with *BamH*I/*Kpn*I; 1–36 bp *nleA* gene with its own promoter amplified from ZAP193 and inserted (Roe *et al.*, 2007)
pDW-12aaNleA	pAJR104 digested with *BamH*I/*Kpn*I; 1–36 bp *nleA* gene with its own promoter amplified from ZAP193 and inserted
pDW37	pET21d digested with *Xba*I/*Xho*I; fragment contained full length *cesT* gene amplified from ZAP193 and inserted
pDW38	pGEX-4T-2 digested with *BamH*I/*Sma*I; fragment contained full length *escF* gene amplified from ZAP193 and inserted
pDW39	pGEX-4T-2 digested with *BamH*I/*Sma*I; fragment contained full length *escD* gene amplified from ZAP193 and inserted
pDW6m-35	pDW6: a synonymous change was introduced into C-terminal *sepL* using site-directed mutagenesis which altered its mRNA structure

pDW6m+1	pDW6: a single base change introduced at +1 site of *sepL* transcript using site-directed mutagenesis
pDW6m	pAJR70 digested with *BamH*I/*Kpn*I; *sepL* gene amplified from ZAP193, cloned in frame 5' to *gfp*
pDW6m0	pDW6m: *sepL*/LEE4 promoter was amplified from ZAP193 and inserted in front of *sepL* gene
pDW6m1	pDW6m1: 5'UTR of GST was amplified from pGEX4T-2 and inserted between LEE4 promoter and *sepL* gene
pDWsepL3	pAJR70 digested with *BamH*I/*Kpn*I; 1-3 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL6	pAJR70 digested with *BamH*I/*Kpn*I; 1-6 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL9	pAJR70 digested with *BamH*I/*Kpn*I; 1-9 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL12	pAJR70 digested with *BamH*I/*Kpn*I; 1-12 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL15	pAJR70 digested with *BamH*I/*Kpn*I; 1-15 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL18	pAJR70 digested with *BamH*I/*Kpn*I; 1-18 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL21	pAJR70 digested with *BamH*I/*Kpn*I; 1-21 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL24	pAJR70 digested with *BamH*I/*Kpn*I; 1-24 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL27	pAJR70 digested with *BamH*I/*Kpn*I; 1-27 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL30	pAJR70 digested with *BamH*I/*Kpn*I; 1-30 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL33	pAJR70 digested with *BamH*I/*Kpn*I; 1-33 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL36	pAJR70 digested with *BamH*I/*Kpn*I; 1-36 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL39	pAJR70 digested with *BamH*I/*Kpn*I; 1-39 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL42	pAJR70 digested with *BamH*I/*Kpn*I; 1-42 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL45	pAJR70 digested with *BamH*I/*Kpn*I; 1-45 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDWsepL48	pAJR70 digested with *BamH*I/*Kpn*I; 1-48 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW-tir	pAJR70 digested with *BamH*I/*Kpn*I; full length *tir* gene with its own promoter amplified from ZAP193 and inserted

Table 5.2 Bacterial strains and antibodies

Strains	**Details**
AAEC185	*Escherichia coli* K-12 strain
BL21 (DE3)	F^- *ompT hsdS$_B$ gal dcm* (DE3)
ZAP193	*Escherichia coli* O157:H7 *stx*-, NCTC 12900
ZAP198	*Escherichia coli* O157:H7 (Naylor *et al.*, 2003)
ZAP108	*Escherichia coli* O157:H7 (Roe *et al.*, 2003b)
ZAP632	ZAP198: *escRSTU* replaced with kanamycin resistance gene
ZAP984	ZAP198: LEE4 replaced with kanamycin resistance gene (Naylor *et al.*, 2005)
ZAP928	ZAP193: *sepL* replaced with *sepL-gfp* (full-length)
ZAP1004	*Escherichia coli* O157 *stx*- Nal^r Δ*ler* (Low *et al.*, 2006)
ZAP1143	*Escherichia coli* NCTC 12900; O157 *stx*- Nal^r Δ*sepL* (total deletion)
ZAP1144	*Escherichia coli* NCTC 12900; O157 *stx*- Nal^r Δ*sepD* (total deletion)
ZAP1211	*Escherichia coli* NCTC 12900; O157 *stx* Nal^r Δ*sepL* (Frameshift mutation by insertion of an additional base at nucleotide position 345 of *sepL*)
ZAP193Δ*escN*	*Escherichia coli* O157:H7 *stx*- Δ*escN*, NCTC 12900 (Roe *et al.*, 2007)
EDL933	*Escherichia coli* O157:H7 sequenced strain (Perna *et al.*, 2001)
ZAP1205	EDL933: *hfq* replaced with chloramphenicol resistance gene
Δ*CsrA*	ZAP193: *csrA* replaced with *sacB-kana^R* cassette
SK	ZAP193: *sepL* replaced with *sacB-kana^R* cassette
M10	ZAP193: the first 10 bases of *sepL* ORF replaced with GGTACC and 3 bases changed in *espA* ORF (279:T/C; 336:G/A; 393:G/A)
Media	**Details**
LB	Luria-Bertani broth, Oxoid. (10 g Bacto tryptone, 5 g Bacto yeast, 10 g NaCl, add deionized H_2O to 1 Liter, adjust pH to 7.0 with NaOH, Sterilize by autoclaving)
M9	M9 minimal medium was modified with a final glycerol concentration of 0.4%, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, MEM Non-Essential Amino acids Solution (Sigma), MEM Amino acids Solution (Sigma).
MEM-HEPES	Minimal essential medium with 25 mM HEPES buffer (Sigma), Glucose was

	added to MEM-HEPES to give a final concentration of 0.2%.
NYZ+	10 g of NZ amine (casein hydrolysate); 5 g of yeast extract; 5 g of NaCl; add deionized H_2O to 1 Liter, adjust pH to 7.5 with NaOH, Sterilize by autoclaving; Add the following filer-sterilized supplements (12.5 ml of 1 M $MgCl_2$; 12.5 ml of 1 M $MgSO_4$ and 10 ml of 2 M glucose) prior to use.
Antibiotics	Antibiotics were included when required at the following concentrations: chloramphenicol (Cam) 12.5 μg ml^{-1}, kanamycin (Kan) 25 μg ml^{-1}, Ampicillin (Amp) 50 μg ml^{-1}
Antibodies	**Details**
Anti GFP	Mouse monoclonal (Clontech)
Anti EspA	Mouse monoclonal (gift from Prof. Trinad Chakraborty)
Anti EspD	Mouse monoclonal (gift from Prof. Trinad Chakraborty)
Anti Tir	Mouse monoclonal (gift from Prof. Trinad Chakraborty)
Anti OmpA	Rabbit polyclonal (gift from Prof. John Leong)
Anti GroEL	Rabbit polyclonal (Stressgen)
Anti-His	Mouse Penta-His antibody (Qiagen)
Anti-Rabbit Igs	Peroxidase-conjugated Swine Anti-Rabbit Immunoglobulins, mainly IgG, HRP (DAKO)
Anti-Mouse Igs	Polyclonal Goat Anti-Mouse Immunoglobulins, mainly IgG, HRP (DAKO)
Anti-Rabbit Igs, FITC/TRITC conjugated	Goat Anti-Rabbit IgG, FITC/TRITC (DAKO)
Alexa Fluor Anti-Mouse IgG	Alexa Fluor 488 goat anti-mouse (Green) ; Alexa Fluor 594 goat anti-mouse (Red) (Invitrogen)

Table 5.3 Oligonucleotides used in the study

Primer Name	Application	Sequence
sepLfull-5'	pDW6,24,26,27,28, 29,30,45,46,47,48, 61,62, pDWsepL3-48	CGGGATCCATGGCTAATGGTATTGAATTTAATCTTACCAGATGCTTGCTTTATTG
sepLfull-3'	pDW6	GGGGTACCAATAATTTCCTCCTTATAGTC
sepL 5'*lhs*	pDW8, pDW10	CGGGGTACCTTTTTAAACTCTGATGCCAG
sepL 3'*lhs*	pDW8, pDW10	CGCGGATCCTGGAAACTCACGTAATC
sepL 5'*rhs*	pDW7	TGCTCTAGATATTAATTACTCAATAATTTTTTTG
sepL 3'*rhs*	pDW7	CCAAGCTTAACAATTTTACTTTTTTGTG
sepL 5'g	pDW9	CGGGATCCATGGCTAATGGTATTGAATTTAATC
C48aasepL 5'g	pDW50	CGGGATCCGAAGATAAACATATTTATTATTTTC
sepL 3'g	pDW9,50	CAACCCGGGTCAAATAATTTCCTCCTTATAGTC
sepL 3BamH	pDW24	CGGGATCCTCAAATAATTTCCTCCTTATAGTC
sepD 5'*lhs*	pDW21	GCGAGCTCCAGCGATCTCAGTTTCGATG
sepD 3'*lhs*	pDW21	GCCGGATCCCATACATATTACCCGTCCTG
sepD 5'*rhs*	pDW22	GCGGATCCCCGCCAACACACTTGTTTTC
sepD 3'*rhs*	pDW22	GCATTAATCGGTCTTTTACAACAACTGC
sepD 5'g	pDW15	CGGGATCCATGAACAATAATAATGGCATAG
sepD 3'g	pDW15	CCCCGGGTTACACAATTCGTCCTATATCAG
sepD 5'	pDW20	CGGGATCCCTAAAGAAAGAGAAAAATGCG
sepD 3'	pDW20	GGGGTACCTTACACAATTCGTCCTATATCAG
cesT 5'g	pDW40	CGGGATCCATGTCATCAAGATCTGAACTTTTA
cesT 3'g	pDW40	CCCCGGGTTATCTTCCGGCGTAATAATGTTTA
sepL51-3'	pDW26	GGGGTACCAGAATTAAAAACAGATGCGGGG

sepL81-3'	pDW61	GGGGTACCCAATTAACGCAAAAAAATTCTTC
sepL84-3'	pDW62	GGGGTACCTTAACGCAAAAAAATTCTTCTAAT
sepL210-3'	pDW27	GGGGTACCACCTTTGCGATATCCCAGGC
sepL573-3'	pDW28	GGGGTACCAGCCTTTTCATAAAGCTTCTTG
sepL870-3'	pDW29	GGGGTACCTGTTAGCCAGACATGTTCAATA
sepL1020-3'	pDW30	GGGGTACCAATCATTAATAATGCATTCTCTC
sepL801-h3	pDW45	CGGGATCCTCAGTGGTGGTGGTGGTGGTGGATCGAAATAATATCTGCATAGT
sepL870-h3	pDW46	CGGGATCCTCAGTGGTGGTGGTGGTGGTGTGTTAGCCAGACATGTTCAATA
sepL1020-h3	pDW47	CGGGATCCTCAGTGGTGGTGGTGGTGGTGAATCATTAATAATGCATTCTCTCT
sepLFL-h3	pDW48	CGGGATCCTCAGTGGTGGTGGTGGTGGTGAATAATTTCCTCCTTATAGTCGA
tir5h	pTir-His,p100Tir-His,p200Tir-His	AAAAATCTAGAAAAGGAGATATTTATGCCTATTG
tir3h	pTir-His,p-200Tir-His,p-382Tir-His	AAAAACTCGAGGACGAAACGATGGGATCCCGGCG
100tir3h	p100Tir-His	CCGCTCGAGGTTAAGAGTATCGAGCGGAC
200tir3h	p200Tir-His	CCGCTCGAGAACGCCTTTTGACTCCCCAG
-200tir5h	p-200Tir-His	GGAATTCCATATGGGGGAGTTGAGGGAGTC
-382tir5h	p-382Tir-His	GGAATTCCATATGCTTCATCGAAAAAATCAGCCG
sepL5h	pDW42	GCTCTAGATGGAATATTCATAATTAATGATTAC
sepL3h	pDW42	CCGCTCGAGTCAAATAATTTCCTCCTTATAGTC
sepL5'*allf*	pDW51	GGGGTACCGACATCATTAATTGCGGATATG
sepL3'*allf*	pDW51	GCTCTAGAGACGAGTTCATGGATTTAACC
sepLfs5	pDW52	GCGGGCTGCTGGAACAACACGACTCCTACTTTGGCGATGTTTGGC
sepLfs3	pDW52	GCCAAACATCGCCAAAGTAGGAGTCGTGTTGTTCCAGCAGCCCGC
sepL-ATG5'	pDW14	CGGGATCCATGGCTAATGGTATTGA

		ATTTAATC
gfp-3'	pDW14	CGGGATCCGGTAATGGTAGCGACCGGCGC
gst5UTR-sepL	pDW6m1	GGTACCTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGTATTCATGGCTAATGGTATTGAATTTAATC
hfq-SD5'	pDW63	CGGGATCCGCATATAAGGAAAAGAGAGAATG
Hfq-3'	pDW63	CGGAATTCTTATTCGGTTTCTTCGCTGTC
csrA-SD5'	pDW66	CGGGATCCTAATCTTTCAAGGAGCAAAGAATG
csrA-3'	pDW66	CGGAATTCTTAGTAACTGGACTGCTGGG
csrARF5	pDW53	CGGGATCCTCTTTCCGCGTCTCATCTTTA
csrARF3	pDW53	TGCATTAATGTTAATTCCGCTACCTCTTCG
csrALF5	pDW54	GGGGTACCAATTTGCCGATCGAAACCAAC
csrALF3	pDW54	CGGGATCCTCTTTGCTCCTTGAAAGATTA
nleA5Xba	pDWNleA-His	GCTCTAGAATGGATATAAACATGTAATAAG
nleA3Xho	pDWNleA-His	CCGCTCGAGTCTTGTTTCTTGGATTATATC
nleA5Nde	pDWHis-NleA	GGAATTCCATATGATGAACATTCAACCGACCATAC
nleA3Xho2	pDWHis-NleA	CCGCTCGAGGACTCTTGTTTCTTGGATTATATC
sepL3	pDWsepL3	GGGGTACCATGGTACCGGTCGCCACCATG
sepL6	pDWsepL6	GGGGTACCGTACCGGTCGCCACCATGGTG
sepL9	pDWsepL9	GGGGTACCCCGGTCGCCACCATGGTGAGC
sepL12	pDWsepL12	GGGGTACCGTCGCCACCATGGTGAGCAAG
sepL15	pDWsepL15	GGGGTACCGCCACCATGGTGAGCAAGGGC
sepL18	pDWsepL18	GGGGTACCACCATGGTGAGCAAGGGCGAG
sepL21	pDWsepL21	GGGGTACCATGGTGAGCAAGGGCGAGGAG
sepL24	pDWsepL24	GGGGTACCGTGAGCAAGGGCGAGGAGCTG
sepL27	pDWsepL27	GGGGTACCAGCAAGGGCGAGGAGCTGTTC
sepL30	pDWsepL30	GGGGTACCAAGGGCGAGGAGCTGTTCACC

sepL33	pDWsepL33	GGGGTACCGGCGAGGAGCTGTTCACCGGG
sepL36	pDWsepL36	GGGGTACCGAGGAGCTGTTCACCGGGGTG
sepL39	pDWsepL39	GGGGTACCAGCTGTTCACCGGGGTGGTG
sepL42	pDWsepL42	GGGGTACCGTTCACCGGGGTGGTGCCC
sepL45	pDWsepL45	GGGGTACCCACCGGGGTGGTGCCCATC
sepL48	pDWsepL48	GGGGTACCGGGTGGTGCCCATCCTG
Tir-pro-Bgl	pDW-tir	GAAGATCTTATGAAATGTTTTATTAAGATTTCAC
Tir-3-Kpn	pDW-tir	GGGGTACCGACGAAACGATGGGATCCC
ptac 5'r	pDW17	GGAAGATCTGGAAGCTGTGGTATGGCTGT
ptac 3'r	pDW17	CGGGGTACCCATCCCGGGTGTTTCCTGTG
sepD 5'r	pDW20	TCCCCCCGGGGTGTTGTTATCAAGTCATCC
sepD 3'r	pDW20	CGGGGTACCCACAATTCGTCCTATATCAG

Chapter 6

General Discussion

For enterohaemorrhagic *Escherichia coli*, a fully functional T3SS is required for inducing typical attaching and effacing lesions on host cells which are crucial for pathogen colonization in either the human patient or natural host reservoir (McDaniel and Kaper, 1997, Naylor *et al.*, 2005). The colonization of EHEC O157 causes pathological changes to epithelial cells in the gastrointestinal tract which include rearrangement of the cytoskeleton and actin accumulation beneath the bacteria and eukaryotic membrane (Knutton *et al.*, 1989b, Frankel *et al.*, 1998). In order to achieve those changes, bacterial need to transfer virulence factors into host cells via a T3SS channel (Kenny *et al.*, 1997b, Abe *et al.*, 1998, McNamara and Donnenberg, 1998, Tomson *et al.*, 2005, Caron *et al.*, 2006). Recent studies have demonstrated that effector proteins can have contradictory functions (Kenny *et al.*, 2002, Tu *et al.*, 2003, Berger *et al.*, 2009), therefore, it is very important to get the effectors delivered to the right place and at the right time for optimal function.

Several mechanisms have evolved to tackle this issue at the transcriptional, posttranscriptional and posttranslational levels in T3SS pathogens (Chilcott and Hughes, 2000, Karlinsey *et al.*, 2000a, Urbanowski *et al.*, 2005, Yahr and Wolfgang, 2006, Lee and Hughes, 2006, Urban and Vogel, 2007, Zarivach *et al.*, 2008, Riordan and Schneewind, 2008). As T3SS expression is the first step of virulence delivery, it has been shown that there are multiple layers of regulation involved in this early stage (McCaw *et al.*, 2002, Urbanowski *et al.*, 2005, Vakulskas *et al.*, 2009, Karlinsey *et al.*, 2000a). Transcriptional activation of T3SS needs various environmental signal inputs which include temperature, calcium/magnesium/ferric/bicarbonate ion concentration changes and cell

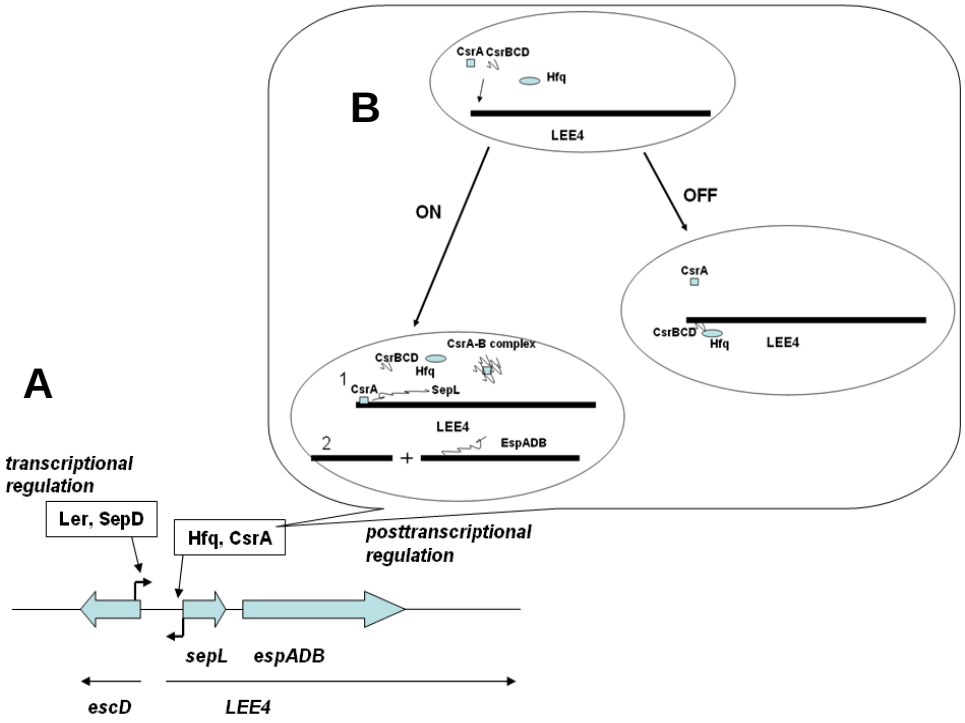


Fig. 6.1. Model for control of *LEE4* expression by Ler, SepD and Hfq/CsrA pathways. SepD and Ler, the global regulator of T3SS, are required to fully activate LEE4 transcription in EHEC O157 (A). Hfq and another regulator-CsrA were found to be important for posttranscriptional regulation of LEE4. The proposed Hfq/CsrA regulation model is shown in B. LEE4 translation is activated when CsrA binds to LEE4 transcript and/or inhibits Hfq mediated CsrB sequestration via CsrA/CsrB binding ('ON'). The large LEE4 (*sepL-espADB*) mRNA is cleaved by RNase E after SepL is translated and then EspADB translation is initiated. LEE4 expression is repressed when CsrB binds to LEE4 transcript with its chaperone Hfq ('OFF'). The Large LEE4 mRNA is sequestered and no translation is initiated.

contact (Kenny *et al.*, 1997a, Beltrametti *et al.*, 2000, Abe *et al.*, 2002, Rosenshine *et al.*, 1996a, Nisan *et al.*, 1998). In this process, EHEC senses the presence of target epithelial cells by detecting multiple host signals and then initiates T3SS expression (Kenny *et al.*, 1997a). According to previous reports, T3SS signal sensor/receptors have been found in other pathogens (Aldon *et al.*, 2000), although the signal sensor(s) has not yet been identified in EHEC. T3SS

activation is known to occur primarily through the global regulator Ler (Mellies *et al.*, 1999, Friedberg *et al.*, 1999, Sperandio *et al.*, 2000, Bustamante *et al.*, 2001, Haack *et al.*, 2003, Berdichevsky *et al.*, 2005, Laaberki *et al.*, 2006). Previously Ler was shown to regulate the transcription level of LEE1, LEE2, LEE3, LEE5 (*tir*) and LEE4 (*esp*) in EHEC and most of this control has been demonstrated to be through direct binding of Ler to the respective promoter regions (Sperandio *et al.*, 2000, Berdichevsky *et al.*, 2005, Haack *et al.*, 2003). Although *sepL* is located adjacent to ORFs encoding EspADB within the LEE4 operon, the transcriptional level of *sepL* and *espADB* were observed to be very different (Kresse *et al.*, 2000, Roe *et al.*, 2003b) and a later study suggested a posttranscriptional mechanism was important for expression of LEE4 (Roe *et al.*, 2003b, Roe *et al.*, 2004, Lodato and Kaper, 2009). In my study, SepL expression was, for the first time, demonstrated to be heterogeneous and co-ordinated with EspA filament expression on the surface of the cell. My data also showed that SepL expression was also activated by Ler, probably at the transcriptional level. This result confirmed that Ler functions as a global regulator for T3SS in EHEC O157:H7. Apart from Ler regulation of SepL production, both SepD and EscRSTU were found to stimulate SepL expression in my work. This could be the result of feedback mechanisms when mutations in the assembly or regulation of T3S are introduced.

6.1 Transcriptional regulation of LEE4

It was known the expression of EHEC T3SS was mainly activated through Ler (Friedberg *et al.*, 1999, Sperandio *et al.*, 2000, Elliott *et al.*, 2000). The level of T3 secretion was found to be variable in certain EHEC strains but not through differences in LEE1 (Ler) expression (Roe *et al.*, 2003b). Although both LEE4 promoter sequences and LEE4 transcription levels were found to be very similar, it was shown that *espADB* transcript levels were different in different EHEC strains (Roe *et al.*, 2003b, Roe *et al.*, 2004). Therefore, it was proposed that LEE4 expression was post-transcriptionally regulated. In my study, to investigate SepL expression, a GFP reporter was tagged to the SepL C-terminus with which SepL expression could be visualized and measured using fluorescence microscopy and fluorimetry. SepL heterogeneous expression was also visualised using this approach. Northern blotting analysis using both *sepL* and *gfp* probes suggested that there were two different LEE4 transcripts detected which might be associated with SepL heterogeneous expression. The minimal sequence requirement for SepL heterogeneity was mapped to the first 81bp of *sepL* ORF using both GFP fusion and RNA modelling. It was observed that the AUG start codon and the ribosome binding site of sepL can be present at the base of harpin loops when modelled using RNA structure software.

6.2 Posttranscriptional regulation of LEE4

Although it was demonstrated that the LEE4 transcript is subject to post-transcriptional processing and the *m*RNA structure is crucial for this regulation, the actual regulator(s) were not known (Roe *et al.*, 2003b, Lodato and Kaper, 2009, Bhatt *et al.*, 2009). In my study, multiple regulators were tested and two

factors were identified. Hfq, a well described sRNA chaperone which is known to be involved in sRNA regulation, was shown to change SepL expression dramatically by >100 fold when Hfq was mutated and it also changed SepL heterogeneous expression. While my work was submitted for publication, two other reports were published which showed Hfq regulation of LEE expression via Ler but without further mechanism (Shakhnovich *et al.*, 2009, Hansen and Kaper, 2009). From my study, it is clear that Ler can activate SepL expression by 12.5 fold. So, considering the impact of Ler regulation, my data implied other Hfq regulation over LEE4 which is not via Ler. Because Hfq is a sRNA chaperone, there are likely to be a sRNAs involved in LEE4/SepL expression via transcript processing/degradation. Another factor which was found to affect SepL expression dramatically in my study was CsrA, an RNA binding protein. It also affected SepL expression not only at the population level but also at the single cell level. A very recent report suggested that CsrA can regulate LEE4 expression via direct binding to the LEE4 transcript (Bhatt *et al.*, 2009). It is known that these two factors were involved in Csr RNA regulation according to previous studies (Romeo, 1996, Romeo, 1998, Babitzke and Romeo, 2007, Lucchetti-Miganeh *et al.*, 2008). Therefore, Csr regulation of LEE4, which includes CsrABCD and Hfq, might be responsible for LEE4 post-transcription regulation with the detailed mechanism still unknown (Fig. 6.1.). Potentially via this mechanism, the expression of the EHEC translocon can be coupled to T3SS assembly which has been indicated for the T3 flagella system (Chilcott and Hughes, 2000, Karlinsey *et al.*, 2000a).

6.3 Posttranslational regulation of T3SS by SepL-SepD complex

6.3.1 SepL-SepD complex controls the substrate specificity of EHEC T3S

So far, my work has shown regulation governing T3SS at two different levels. However, even after the translation of T3SS, there is a posttranslational mechanism employed by EHEC which controls the secretion of T3S proteins. It has been reported that YopN controls effector secretion in *Yersinia* T3SS (Forsberg *et al.*, 1991, Day *et al.*, 2003, Ferracci *et al.*, 2004). YopN was proposed as a syringe plug which blocked the secretion channel of the T3SS. The sensing of low calcium levels triggers release of the T3SS blocker YopN and therefore opens a conduit for effector secretion (Ferracci *et al.*, 2005, Schubot *et al.*, 2005). SepL was previously demonstrated as a key protein for translocon and effector export in EHEC that has homology to YopN/TyeA in *Yersinia* spp. (Pallen *et al.*, 2005a). Here, in my study, the function of SepL was analysed. It was discovered that the C-terminal of SepL is not required for translocon export or SepL membrane localization. Of note was the fact that membrane-associated SepL truncates were also found to have the capacity to interact with SepD, indicating that the two phenotypes may be linked. However, the most significant result was that the C-terminus of SepL was shown to be required for effector secretion but not translocon secretion. Further investigation revealed that the C-terminus of SepL is capable of binding to Tir, considered to be the first effector protein translocated into host cells (Mills *et al.*, 2008). A recent publication suggests that Tir secretion is required for the hypersecretion

phenotype in a *sepD* mutant (Thomas *et al.*, 2007). Therefore, a secretion controlling mechanism is proposed as depicted in Fig.6.2. As LEE4 (*espADB*) and LEE5 (*tir*) are co-ordinately expressed, EspADB and Tir are present in the bacterial cytoplasm at the same time. In my model, effector secretion is held back via SepL-Tir sequestration while EspA filament assembly is ongoing. SepL is important for this model as it acts as a substrate switch which changes T3S from translocon export to effector translocation. Recently it was demonstrated that Ca^{2+} is involved in the regulation of type 3 secretion in EPEC/EHEC/CR (Ide *et al.*, 2003, Deng *et al.*, 2005), altering the ratio between secreted Esp proteins and Tir. This effector oversecretion phenotype was also observed in Δ*sepL* and Δ*sepD* strains. It has been shown that the calcium concentration is much lower in eukaryotic cytoplasm comparing with in bacterial cytoplasm, Therefore, the depletion of Ca^{2+} was considered as a host cell signal and also a symbol of T3 channel opening between the bacterium and the host cell and which somehow alters the SepL-SepD complex conformation and initiates an effector substrate specific secretion. Although the mechanism of how SepL releases Tir is still unknown, analysis of SepL homologue protein structures offers a possibility. MxiC, a structural homologue in *Shigella*, was shown to have a flexible C terminus and structural change could result in substantial domain re-arrangement, opening up one face of the molecule (Deane *et al.*, 2008). Therefore, the same kind of structural change in SepL might be stimulated by changes in calcium concentration or pH levels on contact with the host cell leading to Tir release.

SepD, a binding partner protein of SepL, is also involved in this secretion controlling mechanism. SepD is encoded by a gene (*rorf6*) on the LEE2 operon and it was known that SepD is a membrane-associated protein which binds SepL forming a secretion switch complex (Deng *et al.*, 2005). In my study, SepD was found important not only for binding SepL to form a SepL-SepD switch complex, but also for SepL expression. SepD was suggested as an expression regulator for NleI before (Li *et al.*, 2006). It was also demonstrated that SepD is necessary for normal SepL transcription in my study. Although it was known that the SepL-SepD complex controls the substrate specificity of EHEC T3S, the role of SepD was unknown. In my study, it is implied that SepD binding to SepL was crucial for SepL membrane localization as mentioned above. A previous report, and my own data, indicated that translocon proteins can be exported at extremely low levels from a *sepD* mutant while they cannot be exported at all from a *sepL* mutant (Fig. 2.1 and Deng *et al.*, 2004). It is suggested that SepL might play a more important role in translocon export and this phenotype might result from random membrane localised SepL without the help of SepD localisation to the T3SS.

6.3.2 Two secretion signals and SepL-SepD are required for effector targeting and secretion regulation

EHEC virulence proteins are delivered via the T3SS channel but the targeting mechanism to the T3 basal apparatus is not clear. For T3 effector proteins in *Yersina*, there were two secretion pathways mechanism proposed (Cheng *et al.*, 1997). *Yersinia* effectors have two different secretion signals, an N-terminal signal and a chaperone binding domain (CBD) signal. The N-terminal signal

could target itself to the T3 basal proteins in a chaperone independent way while CBD signal secretion requires a chaperone protein. It has been reported that N-terminal 15aa Tir was sufficient for translocation into host cells via T3SS independent of its chaperone CesT (Crawford and Kaper, 2002). However, nothing was known about the CBD secretion pathway of Tir so far. In my study, these two secretion pathways were investigated and a model proposed for EHEC O157 (Chapter4, Box 1). Tir without its N-terminal 20aa was tested for CBD secretion pathway in my model. My data demonstrated that Tir had a CBD secretion pathway which is enhanced in a *sepL* or *sepD* mutant. NleA, another EHEC T3 effector, was also tested for the N-terminal secretion pathway in my work. It was obvious that the N-terminal 12aa of NleA as a fusion to B-lactamase were able to be exported via T3SS in EHEC. Critically, this construct could not be secreted in a *sepL* or *sepD* mutant which normally results in an effector hypersecretion phenotype. As SepL-SepD complex is the controller for effector secretion in EHEC, it led to another hypothesis that the SepL-SepD complex is required for N-terminal signal targeting for secretion via direct interactions. Using GST pull down assays, N-terminal 12aa NleA was found to interact directly with SepD but not SepL. My subsequent Far-Western analysis suggested that SepD could bind most of the hypersecreted effectors but not Tir which interacted with SepL. My GST pull down and Far-Western results strongly suggest that SepL and SepD proteins act together as a T3 gating complex for effector secretion. CesT, which is a key part of effector secretion pathway, is known to interact with T3SS ATPase — EscN with or without Tir (Gauthier and Finlay, 2003), However it was found still membrane associated in an EscN mutant (Thomas *et al.*, 2005) which suggested another T3 membrane

localised protein interacts with CesT. As the SepL-SepD complex is key to control of effector secretion and membrane-associated, they were both tested for their CesT binding capacity using GST pull downs in my study and CesT binding to SepD *in vitro* was demonstrated. Therefore, taking all the

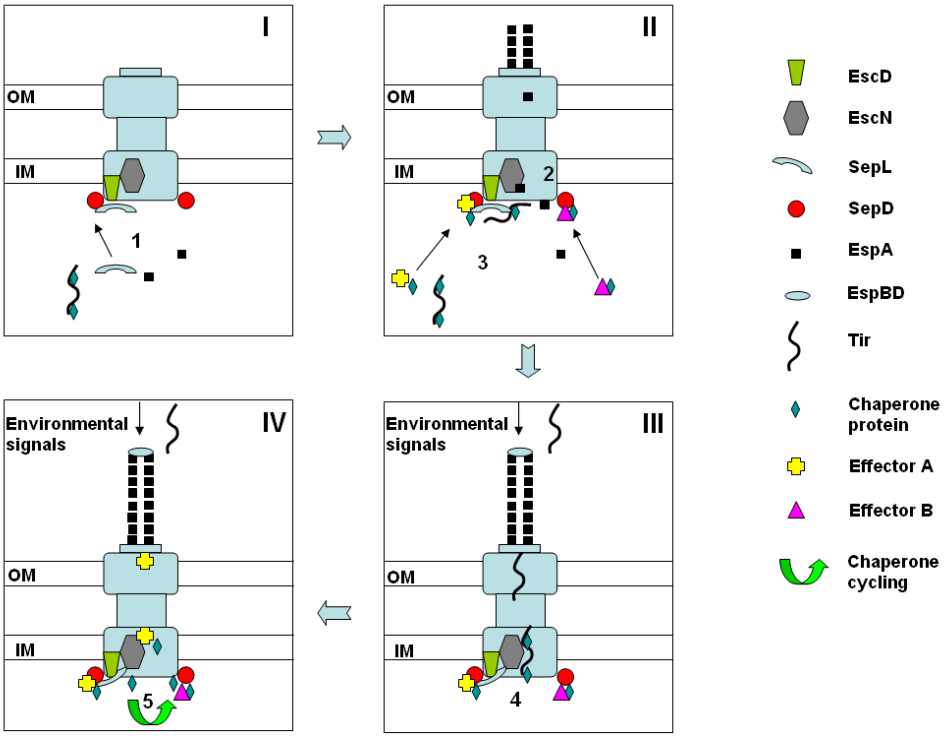


Fig. 6.2. Posttranslational regulation of T3 translocon assembly and effector secretion.
A secretion control model is proposed based on my studies of the SepL-SepD complex. (1) SepL localises to T3 basal apparatus in a SepD dependent manner; (2) SepL holds back effector secretion via Tir interaction while facilitating EspA filament production; (3) effectors target to SepL-SepD complex via SepD binding; (4) after sensing host cells signals, SepL structure changes to release Tir and unblock T3 channel; (5) effector protein secretion via a chaperone cycling mechanism between the SepD-SepL complex and the ATPase, EscN.

information together, a model (Fig.6.2) is proposed for the role of SepL-SepD complex: 1) SepL localizes to the T3 basal apparatus in a SepD-dependent manner; 2) SepL holds up effector secretion via Tir binding while facilitating

EspA filament production; 3) effectors are targeted to the T3SS via the SepL-SepD complex; 4) after sensing host cell signals, the SepL structure is altered to release Tir possibly via an interaction with EscD; 5) effector protein secretion then occurs via a chaperone cycling mechanism based on CesT.

My study of the SepL-SepD complex reveals that EHEC T3SS requires multiple regulatory inputs to coordinate its expression, assembly and secretion. My work has made a contribution to our understanding of secretion control in EHEC and EPEC. The proposed expression and secretion regulation models also lead to some interesting hypotheses about EHEC/EPEC T3SS which should to be tested in the future.

References:

ABE, A., DE GRADO, M., PFUETZNER, R. A., SANCHEZ-SANMARTIN, C., DEVINNEY, R., PUENTE, J. L., STRYNADKA, N. C. & FINLAY, B. B. (1999) Enteropathogenic *Escherichia coli* translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. *Mol Microbiol,* 33, 1162-75.

ABE, A., HECZKO, U., HEGELE, R. G. & BRETT FINLAY, B. (1998) Two enteropathogenic *Escherichia coli* type III secreted proteins, EspA and EspB, are virulence factors. *J Exp Med,* 188, 1907-16.

ABE, H., MIYAHARA, A., OSHIMA, T., TASHIRO, K., OGURA, Y., KUHARA, S., OGASAWARA, N., HAYASHI, T. & TOBE, T. (2008) Global regulation by horizontally transferred regulators establishes the pathogenicity of *Escherichia coli*. *DNA Res,* 15, 25-38.

ABE, H., TATSUNO, I., TOBE, T., OKUTANI, A. & SASAKAWA, C. (2002) Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun,* 70, 3500-9.

ADLERBERTH, I., CARLSSON, B., DE MAN, P., JALIL, F., KHAN, S. R., LARSSON, P., MELLANDER, L., SVANBORG, C., WOLD, A. E. & HANSON, L. A. (1991) Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand,* 80, 602-10.

AGRAIN, C., CALLEBAUT, I., JOURNET, L., SORG, I., PAROZ, C., MOTA, L. J. & CORNELIS, G. R. (2005) Characterization of a Type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. *Mol Microbiol,* 56, 54-67.

AKASHI, S., JOH, K., TSUJI, A., ITO, H., HOSHI, H., HAYAKAWA, T., IHARA, J., ABE, T., HATORI, M., MORI, T. & NAKAMURA, T. (1994) A severe outbreak of hemorrhagic colitis and hemolytic syndrome-associated with *Escherichia coli* O157:H7 in Japan. *Eur J Pediatr,* 153, 650-655.

AKEDA, Y. & GALAN, J. E. (2005) Chaperone release and unfolding of substrates in type III secretion. *Nature,* 437, 911-5.

ALDON, D., BRITO, B., BOUCHER, C. & GENIN, S. (2000) A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. *EMBO J,* 19, 2304-2314.

ALDRIDGE, P. & HUGHES, K. T. (2001) How and when are substrates selected for type III secretion? *Trends Microbiol,* 9, 209-14.

ALDRIDGE, P., KARLINSEY, J. & HUGHES, K. T. (2003) Type III secretion chaperone FlgN regulates flagellar assembly via a negative feedback loop containing its chaperone substrates FlgK and FlgL. *Mol Microbiol,* 49, 1333-1345.

ALDRIDGE, P., KARLINSEY, J. E., BECKER, E., CHEVANCE, F. F. & HUGHES, K. T. (2006) Flk prevents premature secretion of the anti-sigma factor FlgM into the periplasm. *Mol Microbiol,* 60, 630-43.

ALJADER, L., SALMON, R. L., WALKER, A. M., WILLIAMS, H. M., WILLSHAW, G. A. & CHEASTY, T. (1999) Outbreak of *Escherichia coli* O157 in a nursery: lessons for prevention. *Arch Dis Child,* 81, 60-63.

ALLEN-VERCOE, E., WADDELL, B., TOH, M. C. & DEVINNEY, R. (2006) Amino acid residues within enterohemorrhagic *Escherichia coli* O157:H7 Tir involved in phosphorylation, alpha-actinin recruitment, and Nck-independent pedestal formation. *Infect Immun,* 74, 6196-205.
ANAGNOU, N. P., PAPANICOLAOU, N. & FESSAS, P. (1991) Recurrent attacks of hemolytic uremic syndrome. *Haematologia (Budap),* 24, 101-5.
ANDERSON, D. M. & SCHNEEWIND, O. (1997) A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science,* 278, 1140-3.
ANDRADE, A., PARDO, J. P., ESPINOSA, N., PEREZ-HERNANDEZ, G. & GONZALEZ-PEDRAJO, B. (2007) Enzymatic characterization of the enteropathogenic *Escherichia coli* type III secretion ATPase EscN. *Arch Biochem Biophys,* 468, 121-7.
ARMSTRONG, G. L., HOLLINGSWORTH, J. & MORRIS, J. G., JR. (1996) Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol Rev,* 18, 29-51.
ARTZ, R. R. & KILLHAM, K. (2002) Survival of *Escherichia coli* O157:H7 in private drinking water wells: influences of protozoan grazing and elevated copper concentrations. *FEMS Microbiol Lett,* 216, 117-22.
ATTENBOROUGH, M. & MATTHEWS, K. R. (2000) Food safety through the meat supply chain. *Symp Ser Soc Appl Microbiol*, 144S-148S.
AUVRAY, F., THOMAS, J., FRASER, G. M. & HUGHES, C. (2001) Flagellin polymerisation control by a cytosolic export chaperone. *J Mol Biol,* 308, 221-9.
BABITZKE, P. & ROMEO, T. (2007) CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol,* 10, 156-63.
BADEA, L., BEATSON, S. A., KAPARAKIS, M., FERRERO, R. L. & HARTLAND, E. L. (2009) Secretion of flagellin by the LEE-encoded type III secretion system of enteropathogenic *Escherichia coli*. *BMC Microbiol,* 9, 30.
BALDINI, M. M., KAPER, J. B., LEVINE, M. M., CANDY, D. C. & MOON, H. W. (1983a) Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. *J Pediatr Gastroenterol Nutr,* 2, 534-8.
BALDINI, M. M., KAPER, J. B., LEVINE, M. M. & MOON, H. W. (1983b) Molecular nature of adhesion in enteropathogenic *Escherichia coli*. *Lancet,* 2, 218.
BAR TANA, J., HOWLETT, B. J. & KOSHLAND, D. E., JR. (1977) Flagellar formation in *Escherichia coli* electron transport mutants. *J Bacteriol,* 130, 787-92.
BARBA, J., BUSTAMANTE, V. H., FLORES-VALDEZ, M. A., DENG, W., FINLAY, B. B. & PUENTE, J. L. (2005) A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. *J Bacteriol,* 187, 7918-30.
BASINENI, S. R., MADHUGIRI, R., KOLMSEE, T., HENGGE, R. & KLUG, G. (2009) The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target rpoS in *Escherichia coli* is growth phase dependent. *RNA Biol,* 6, 584-94.
BAUCHART, P., GERMON, P., BREE, A., OSWALD, E., HACKER, J. & DOBRINDT, U. (2010) Pathogenomic comparison of human extraintestinal and avian pathogenic *Escherichia coli* - Search for factors involved in host specificity or zoonotic potential. *Microb Pathog,* 49, 105-115.

BEINKE, C., LAARMANN, S., WACHTER, C., KARCH, H., GREUNE, L. & SCHMIDT, M. A. (1998) Diffusely adhering *Escherichia coli* strains induce attaching and effacing phenotypes and secrete homologs of Esp proteins. *Infect Immun,* 66, 528-539.
BELTRAMETTI, F., KRESSE, A. U. & GUZMAN, C. A. (1999) Transcriptional regulation of the esp genes of enterohemorrhagic *Escherichia coli*. *J Bacteriol,* 181, 3409-18.
BELTRAMETTI, F., KRESSE, A. U. & GUZMAN, C. A. (2000) Transcriptional regulation of the *pas* gene of enterohemorrhagic *Escherichia coli*. *FEMS Microbiol Lett,* 184, 119-25.
BENDER, J. B., HEDBERG, C. W., BESSER, J. M., BOXRUD, D. J., MACDONALD, K. L. & OSTERHOLM, M. T. (1997) Surveillance by molecular subtype for *Escherichia coli* O157:H7 infections in Minnesota by molecular subtyping. *N Engl J Med,* 337, 388-94.
BERDICHEVSKY, T., FRIEDBERG, D., NADLER, C., ROKNEY, A., OPPENHEIM, A. & ROSENSHINE, I. (2005) Ler is a negative autoregulator of the LEE1 operon in enteropathogenic *Escherichia coli*. *J Bacteriol,* 187, 349-57.
BERG, H. C. (2003) The rotary motor of bacterial flagella. *Annu Rev Biochem,* 72, 19-54.
BERGER, C. N., CREPIN, V. F., JEPSON, M. A., ARBELOA, A. & FRANKEL, G. (2009) The mechanisms used by enteropathogenic *Escherichia coli* to control filopodia dynamics. *Cell Microbiol,* 11, 309-22.
BERTIN, P., HOMMAIS, F., KRIN, E., SOUTOURINA, O., TENDENG, C., DERZELLE, S. & DANCHIN, A. (2001) H-NS and H-NS-like proteins in Gram-negative bacteria and their multiple role in the regulation of bacterial metabolism. *Biochimie,* 83, 235-41.
BESSER, R. E., LETT, S. M., WEBER, J. T., DOYLE, M. P., BARRETT, T. J., WELLS, J. G. & GRIFFIN, P. M. (1993) An Outbreak of Diarrhea and Hemolytic Uremic Syndrome From *Escherichia coli* O157:H7 in Fresh-Pressed Apple Cider. *JAMA,* 269, 2217-2220.
BHATT, S., EDWARDS, A. N., NGUYEN, H. T., MERLIN, D., ROMEO, T. & KALMAN, D. (2009) The RNA binding protein CsrA is a pleiotropic regulator of the locus of enterocyte effacement pathogenicity island of enteropathogenic *Escherichia coli*. *Infect Immun,* 77, 3552-68.
BINGLE, L. E., BAILEY, C. M. & PALLEN, M. J. (2008) Type VI secretion: a beginner's guide. *Curr Opin Microbiol,* 11, 3-8.
BIRTALAN, S. & GHOSH, P. (2001) Structure of the *Yersinia* type III secretory system chaperone SycE. *Nat Struct Biol,* 8, 974-8.
BIRTALAN, S. C., PHILLIPS, R. M. & GHOSH, P. (2002) Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. *Mol Cell,* 9, 971-980.
BLOMFIELD, I. C., VAUGHN, V., REST, R. F. & EISENSTEIN, B. I. (1991) Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. *Mol Microbiol,* 5, 1447-57.
BODELON, G., MARIN, E. & FERNANDEZ, L. A. (2009) Role of periplasmic chaperones and BamA (YaeT/Omp85) in folding and secretion of intimin from enteropathogenic *Escherichia coli* strains. *J Bacteriol,* 191, 5169-79.

BOGDANOVIC, R., CVORIC, A., NIKOLIC, V. & SINDJIC, M. (1988) Recurrent haemolytic-uraemic syndrome with hypocomplementaemia: a case report. *Pediatr Nephrol,* 2, 236-8.
BONACORSI, S. & BINGEN, E. (2005) Molecular epidemiology of *Escherichia coli* causing neonatal meningitis. *Int J Med Microbiol,* 295, 373-81.
BOSE, N., PAYNE, S. M. & TAYLOR, R. K. (2002) Type 4 pilus biogenesis and type II-mediated protein secretion by *Vibrio cholerae* occur independently of the TonB-facilitated proton motive force. *J Bacteriol,* 184, 2305-9.
BOTTEAUX, A., SORY, M. P., BISKRI, L., PARSOT, C. & ALLAOUI, A. (2009) MxiC is secreted by and controls the substrate specificity of the *Shigella flexneri* type III secretion apparatus. *Mol Microbiol,* 71, 449-60.
BOYCE, T. G., SWERDLOW, D. L. & GRIFFIN, P. M. (1995) *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *N Engl J Med,* 333, 364-8.
BOYD, B. & LINGWOOD, C. (1989) Verotoxin receptor glycolipid in human renal tisue. *Nephron,* 51, 207-210.
BRADBEER, C. (1993) The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. *J Bacteriol,* 175, 3146-50.
BRAY, J. (1945) Isolation of antigenically homogenous strains of *Bact. coli neapolitanum* from summer diarrhoea in infants. *J Pathol Bacteriol* 57, 239–247.
BRENNAN, P. C., FRITZ, T. E., FLYNN, R. J. & POOLE, C. M. (1965) *Citrobacter freundii* associated with diarrhea in a laboratory mice. *Lab Anim Care,* 15, 266-75.
BRENNER, D. J., FANNING, G. R., JOHNSON, K. E., CITARELLA, R. V. & FALKOW, S. (1969) Polynucleotide sequence relationships among members of Enterobacteriaceae. *J Bacteriol,* 98, 637-50.
BRESCIA, C. C., MIKULECKY, P. J., FEIG, A. L. & SLEDJESKI, D. D. (2003) Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure. *RNA,* 9, 33-43.
BREWSTER, D. H., BROWN, M. I., ROBERTSON, D., HOUGHTON, G. L., BIMSON, J. & SHARP, J. C. (1994) An outbreak of *Escherichia coli* O157 associated with a children's paddling pool. *Epidemiol Infect,* 112, 441-7.
BROMS, J. E., FORSLUND, A. L., FORSBERG, A. & FRANCIS, M. S. (2003) Dissection of homologous translocon operons reveals a distinct role for YopD in type III secretion by *Yersinia pseudotuberculosis*. *Microbiology,* 149, 2615-26.
BUSTAMANTE, V. H., SANTANA, F. J., CALVA, E. & PUENTE, J. L. (2001) Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS- dependent repression. *Mol Microbiol,* 39, 664-678.
BUTTNER, C. R., SORG, I., CORNELIS, G. R., HEINZ, D. W. & NIEMANN, H. H. (2008) Structure of the *Yersinia enterocolitica* type III secretion translocator chaperone SycD. *J Mol Biol,* 375, 997-1012.
CALIER, M. F. P., NIOCHE, P., BROUTIN-L'HERMITE, I., BOUJEMAA, R., LE CLAINCHE, C., EGILE, C., GARBAY, A., DUCRUIX, A., SANSONETTI, P AND PANTALONI, D. (2000) GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. 2000. *J Biol Chem,* 275, 21946 - 21952.
CAMBRONNE, E. D. & SCHNEEWIND, O. (2002) *Yersinia enterocolitica* type III secretion: yscM1 and yscM2 regulate yop gene expression by a

posttranscriptional mechanism that targets the 5' untranslated region of yop mRNA. *J Bacteriol,* 184, 5880-93.
CAMPELLONE, K. G., GIESE, A., TIPPER, D. J. & LEONG, J. M. (2002) A tyrosine-phosphorylated 12-amino-acid sequence of enteropathogenic *Escherichia coli* Tir binds the host adaptor protein Nck and is required for Nck localization to actin pedestals. *Mol Microbiol,* 43, 1227-41.
CAMPELLONE, K. G. & LEONG, J. M. (2003) Tails of two Tirs: actin pedestal formation by enteropathogenic *Escherichia coli* and enterohemorrhagic *Escherichia coli* O157:H7. *Curr Opin Microbiol,* 6, 82-90.
CAMPELLONE, K. G., ROBBINS, D. & LEONG, J. M. (2004) EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev Cell,* 7, 217-28.
CARON, E., CREPIN, V. F., SIMPSON, N., KNUTTON, S., GARMENDIA, J. & FRANKEL, G. (2006) Subversion of actin dynamics by EPEC and EHEC. *Curr Opin Microbiol,* 9, 40-5.
CARROLL, A. M., GIBSON, A. & MCNAMARA, E. B. (2005) Laboratory-based surveillance of human verocytotoxigenic *Escherichia coli* infection in the Republic of Ireland, 2002-2004. *J Med Microbiol,* 54, 1163-9.
CARTER, A. O., BORCZYK, A. A., CARLSON, J. A., HARVEY, B., HOCKIN, J. C., KARMALI, M. A., KRISHNAN, C., KORN, D. A. & LIOR, H. (1987) A severe outbreak of *Escherichia coli* O157:H7--associated hemorrhagic colitis in a nursing home. *N Engl J Med,* 317, 1496-500.
CDC (1982) Isolation of *Escherichia coli* O157:H7 from sporadic cases of hemorrhagic colitis - United States. *MMWR Morb Mortal Wkly Rep,* 31, 580-5.
CDC (1994a) *Escherichia coli* O157:H7 outbreak linked to home-cooked hamburger--California, July 1993. *MMWR Morb Mortal Wkly Rep,* 43, 213-6.
CDC (1994b) From the Centers for Disease Control and Prevention. *Escherichia coli* O157:H7 outbreak linked to home-cooked hamburger--California, July 1993. *JAMA,* 271, 1153-4.
CDC (1995a) Enhanced detection of sporadic *Escherichia coli* O157:H7 infections--New Jersey, July 1994. *MMWR Morb Mortal Wkly Rep,* 44, 417-8.
CDC (1995b) From the Centers for Disease Control and Prevention. Enhanced detection of sporadic *Escherichia coli* O157:H7 infections--New Jersey, July 1994. *JAMA,* 274, 17-19.
CDC (1996a) Lake-associated outbreak of *Escherichia coli* O157:H7--Illinois, 1995. *MMWR Morb Mortal Wkly Rep,* 45, 437-9.
CDC (1996b) Outbreak of *Escherichia coli* O157:H7 infection--Georgia and Tennessee, June 1995. *MMWR Morb Mortal Wkly Rep,* 45, 249-51.
CDC (1997) From the Centers for Disease Control and Prevention. Outbreaks of *Escherichia coli* O157:H7 infection associated with eating alfalfa sprouts--Michigan and Virginia, June-July 1997. *JAMA,* 278, 809-10.
CDC (1999) Outbreak of *Escherichia coli* O157:H7 and *Campylobacter* among attendees of the Washington County Fair-New York, 1999. *MMWR Morb Mortal Wkly Rep,* 48, 803-5.
CDC (2000a) From the Centers for Disease Control. Outbreak of *Escherichia coli* O157:H7 infection associated with eating fresh cheese curds--Wisconsin, June 1998. *JAMA,* 284, 2991-2.

CDC (2000b) Outbreak of *Escherichia coli* O157:H7 infection associated with eating fresh cheese curds--Wisconsin, June 1998. *MMWR Morb Mortal Wkly Rep,* 49, 911-3.
CDC (2005) Outbreaks of *Escherichia coli* O157:H7 associated with petting zoos--North Carolina, Florida, and Arizona, 2004 and 2005. *MMWR Morb Mortal Wkly Rep,* 54, 1277-80.
CDC (2007) *Escherichia coli* O157:H7 infection associated with drinking raw milk--Washington and Oregon, November-December 2005. *MMWR Morb Mortal Wkly Rep,* 56, 165-7.
CDC (2010) Two multistate outbreaks of Shiga toxin--producing *Escherichia coli* infections linked to beef from a single slaughter facility - United States, 2008. *MMWR Morb Mortal Wkly Rep,* 59, 557-60.
CHAO, Y. & VOGEL, J. (2010) The role of Hfq in bacterial pathogens. *Curr Opin Microbiol,* 13, 24-33.
CHAPMAN, P. A., SIDDONS, C. A., MALO, A. T. C. & HARKIN, M. A. (1997) A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiol Infect,* 119, 245-250.
CHAPMAN, P. A., SIDDONS, C. A., MALO, A. T. C. & HARKIN, M. A. (2000) A one year study of *Escherichia coli* O157 in raw beef and lamb products. *Epidemiol Infect,* 124, 207-213.
CHAPMAN, P. A., WRIGHT, D. J., NORMAN, P., FOX, J. & CRICK, E. (1993) Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in Man. *Epidemiol Infect,* 111, 439-447.
CHARKOWSKI, A. O., HUANG, H. C. & COLLMER, A. (1997) Altered localization of HrpZ in *Pseudomonas syringae* pv. syringae *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. *J Bacteriol,* 179, 3866-74.
CHARPENTIER, X. & OSWALD, E. (2004) Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. *J Bacteriol,* 186, 5486-95.
CHASE-TOPPING, M., GALLY, D., LOW, C., MATTHEWS, L. & WOOLHOUSE, M. (2008) Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat Rev Microbiol,* 6, 904-12.
CHEN, S. L., HUNG, C. S., PINKNER, J. S., WALKER, J. N., CUSUMANO, C. K., LI, Z., BOUCKAERT, J., GORDON, J. I. & HULTGREN, S. J. (2009) Positive selection identifies an *in vivo* role for FimH during urinary tract infection in addition to mannose binding. *Proc Natl Acad Sci U S A,* 106, 22439-44.
CHENG, L. W., ANDERSON, D. M. & SCHNEEWIND, O. (1997) Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. *Mol Microbiol,* 24, 757-65.
CHENG, L. W., KAY, O. & SCHNEEWIND, O. (2001) Regulated secretion of YopN by the type III machinery of *Yersinia enterocolitica*. *J Bacteriol,* 183, 5293-301.

CHENG, L. W. & SCHNEEWIND, O. (1999) *Yersinia enterocolitica* type III secretion. On the role of SycE in targeting YopE into HeLa cells. *J Biol Chem,* 274, 22102-8.
CHENG, L. W. & SCHNEEWIND, O. (2000a) Type III machines of Gram-negative bacteria: delivering the goods. *Trends Microbiol,* 8, 214-20.
CHENG, L. W. & SCHNEEWIND, O. (2000b) *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol,* 182, 3183-90.
CHILCOTT, G. S. & HUGHES, K. T. (2000) Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica serovar typhimurium* and *Escherichia coli*. *Microbiol Mol Biol Rev,* 64, 694-708.
CHRISTIE, P. J., ATMAKURI, K., KRISHNAMOORTHY, V., JAKUBOWSKI, S. & CASCALES, E. (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu Rev Microbiol,* 59, 451-85.
CIANCIOTTO, N. P. (2005) Type II secretion: a protein secretion system for all seasons. *Trends Microbiol,* 13, 581-8.
CLEARY, J., LAI, L. C., SHAW, R. K., STRAATMAN-IWANOWSKA, A., DONNENBERG, M. S., FRANKEL, G. & KNUTTON, S. (2004) Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology,* 150, 527-38.
COHEN, M. B. & GIANNELLA, R. A. (1992) Hemorrhagic colitis associated with *Escherichia coli* O157:H7. *Adv Intern Med,* 37, 173-95.
CONWAY, P. L. (1995) Microbial ecology of the human large intestine. In: G.R. Gibson and G.T. Macfarlane, eds. p.1-24. *Human colonic bacteria: role in nutrition, physiology, and pathology. CRC Press, Boca Raton, FL.,* .
COOMBES, B. K., BROWN, N. F., VALDEZ, Y., BRUMELL, J. H. & FINLAY, B. B. (2004) Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. *J Biol Chem,* 279, 49804-15.
CORNELIS, G. R. (2006) The type III secretion injectisome. *Nat Rev Microbiol,* 4, 811-25.
CORNELIS, G. R. & WOLF-WATZ, H. (1997) The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol,* 23, 861-7.
CORRIGAN, J. J., JR. & BOINEAU, F. G. (2001) Hemolytic-uremic syndrome. *Pediatr Rev,* 22, 365-9.
COSAR, G., HOSGOR, M., OZGENC, O., HILMIOGLU, S. & TASLI, H. (2001) Expression of P fimbriae of uropathogenic *Escherichia coli* strains. *Infez Med,* 9, 98-100.
CRANE, J. K., MCNAMARA, B. P. & DONNENBERG, M. S. (2001) Role of EspF in host cell death induced by enteropathogenic *Escherichia coli*. *Cell Microbiol,* 3, 197-211.
CRAWFORD, J. A. & KAPER, J. B. (2002) The N-terminus of enteropathogenic *Escherichia coli* (EPEC) Tir mediates transport across bacterial and eukaryotic cell membranes. *Mol Microbiol,* 46, 855-68.
CREASEY, E. A., DELAHAY, R. M., BISHOP, A. A., SHAW, R. K., KENNY, B., KNUTTON, S. & FRANKEL, G. (2003a) CesT is a bivalent

enteropathogenic *Escherichia coli* chaperone required for translocation of both Tir and Map. *Mol Microbiol,* 47, 209-21.
CREASEY, E. A., DELAHAY, R. M., DANIELL, S. J. & FRANKEL, G. (2003b) Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic *Escherichia coli*. *Microbiology,* 149, 2093-106.
CREASEY, E. A., FRIEDBERG, D., SHAW, R. K., UMANSKI, T., KNUTTON, S., ROSENSHINE, I. & FRANKEL, G. (2003c) CesAB is an enteropathogenic *Escherichia coli* chaperone for the type-III translocator proteins EspA and EspB. *Microbiology,* 149, 3639-47.
CREPIN, V. F., PRASANNAN, S., SHAW, R. K., WILSON, R. K., CREASEY, E., ABE, C. M., KNUTTON, S., FRANKEL, G. & MATTHEWS, S. (2005a) Structural and functional studies of the enteropathogenic *Escherichia coli* type III needle complex protein EscJ. *Mol Microbiol,* 55, 1658-70.
CREPIN, V. F., SHAW, R., KNUTTON, S. & FRANKEL, G. (2005b) Molecular basis of antigenic polymorphism of EspA filaments: development of a peptide display technology. *J Mol Biol,* 350, 42-52.
CROSA, L. M., CROSA, J. H. & HEFFRON, F. (2009) Iron transport in *Francisella* in the absence of a recognizable TonB protein still requires energy generated by the proton motive force. *Biometals,* 22, 337-44.
CROXEN, M. A. & FINLAY, B. B. (2010) Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat Rev Microbiol,* 8, 26-38.
CRUMP, J. A., BRADEN, C. R., DEY, M. E., HOEKSTRA, R. M., RICKELMAN-APISA, J. M., BALDWIN, D. A., DE FIJTER, S. J., NOWICKI, S. F., KOCH, E. M., BANNERMAN, T. L., SMITH, F. W., SARISKY, J. P., HOCHBERG, N. & MEAD, P. S. (2003) Outbreaks of *Escherichia coli* O157 infections at multiple county agricultural fairs: a hazard of mixing cattle, concession stands and children. *Epidemiol Infect,* 131, 1055-62.
CRUMP, J. A., SULKA, A. C., LANGER, A. J., SCHABEN, C., CRIELLY, A. S., GAGE, R., BAYSINGER, M., MOLL, M., WITHERS, G., TONEY, D. M., HUNTER, S. B., HOEKSTRA, R. M., WONG, S. K., GRIFFIN, P. M. & VAN GILDER, T. J. (2002) An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. *N Engl J Med,* 347, 555-60.
CZERUCKA, D., DAHAN, S., MOGRABI, B., ROSSI, B. & RAMPAL, P. (2001) Implication of mitogen-activated protein kinases in T84 cell responses to enteropathogenic *Escherichia coli* infection. *Infect Immun,* 69, 1298-1305.
DANIELL, S. J., KOCSIS, E., MORRIS, E., KNUTTON, S., BOOY, F. P. & FRANKEL, G. (2003) 3D structure of EspA filaments from enteropathogenic *Escherichia coli*. *Mol Microbiol,* 49, 301-8.
DANIELL, S. J., TAKAHASHI, N., WILSON, R., FRIEDBERG, D., ROSENSHINE, I., BOOY, F. P., SHAW, R. K., KNUTTON, S., FRANKEL, G. & AIZAWA, S. (2001) The filamentous type III secretion translocon of enteropathogenic *Escherichia coli*. *Cell Microbiol,* 3, 865-71.
DAY, J. B., FERRACCI, F. & PLANO, G. V. (2003) Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis* yopN, tyeA, sycN, yscB and lcrG deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. *Mol Microbiol,* 47, 807-23.
DAY, J. B. & PLANO, G. V. (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol Microbiol,* 30, 777-88.

DEAN, P. & KENNY, B. (2004) Intestinal barrier dysfunction by enteropathogenic *Escherichia coli* is mediated by two effector molecules and a bacterial surface protein. *Mol Microbiol,* 54, 665-75.
DEAN, P., MARESCA, M., SCHULLER, S., PHILLIPS, A. D. & KENNY, B. (2006) Potent diarrheagenic mechanism mediated by the cooperative action of three enteropathogenic *Escherichia coli*-injected effector proteins. *Proc Natl Acad Sci U S A,* 103, 1876-81.
DEANE, J. E., ROVERSI, P., KING, C., JOHNSON, S. & LEA, S. M. (2008) Structures of the *Shigella flexneri* type 3 secretion system protein MxiC reveal conformational variability amongst homologues. *J Mol Biol,* 377, 985-92.
DEGRANDIS, S., LAW, H., BRUNTON, J., GYLES, C. & LINGWOOD, C. A. (1989) Globotetraosylceramide is recognized by the pig edema disease toxin. *J Biol Chem,* 264, 12520-5.
DEIBEL, C., KRAMER, S., CHAKRABORTY, T. & EBEL, F. (1998) EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Mol Microbiol,* 28, 463-474.
DELAHAY, R. M., KNUTTON, S., SHAW, R. K., HARTLAND, E. L., PALLEN, M. J. & FRANKEL, G. (1999) The coiled-coil domain of EspA is essential for the assembly of the type III secretion translocon on the surface of enteropathogenic *Escherichia coli*. *J Biol Chem,* 274, 35969-35974.
DELAHAY, R. M., SHAW, R. K., ELLIOTT, S. J., KAPER, J. B., KNUTTON, S. & FRANKEL, G. (2002) Functional analysis of the enteropathogenic *Escherichia coli* type III secretion system chaperone CesT identifies domains that mediate substrate interactions. *Mol Microbiol,* 43, 61-73.
DENG, W., LI, Y., HARDWIDGE, P. R., FREY, E. A., PFUETZNER, R. A., LEE, S., GRUENHEID, S., STRYNAKDA, N. C., PUENTE, J. L. & FINLAY, B. B. (2005) Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. *Infect Immun,* 73, 2135-46.
DENG, W., LI, Y., VALLANCE, B. A. & FINLAY, B. B. (2001) Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. *Infect Immun,* 69, 6323-35.
DENG, W., PUENTE, J. L., GRUENHEID, S., LI, Y., VALLANCE, B. A., VAZQUEZ, A., BARBA, J., IBARRA, J. A., O'DONNELL, P., METALNIKOV, P., ASHMAN, K., LEE, S., GOODE, D., PAWSON, T. & FINLAY, B. B. (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc Natl Acad Sci U S A,* 101, 3597-602.
DESVAUX, M., HEBRAUD, M., HENDERSON, I. R. & PALLEN, M. J. (2006) Type III secretion: what's in a name? *Trends Microbiol,* 14, 157-60.
DEVINNEY, R., GAUTHIER, A., ABE, A. & FINLAY, B. B. (1999) Enteropathogenic *Escherichia coli*: a pathogen that inserts its own receptor into host cells. *Cell Mol Life Sci,* 55, 961-976.
DEVINNEY, R., PUENTE, J. L., GAUTHIER, A., GOOSNEY, D. & FINLAY, B. B. (2001) Enterohaemorrhagic and enteropathogenic *Escherichia coli* use a different Tir-based mechanism for pedestal formation. *Mol Microbiol,* 41, 1445-58.

DHAKAL, B. K., KULESUS, R. R. & MULVEY, M. A. (2008) Mechanisms and consequences of bladder cell invasion by uropathogenic *Escherichia coli*. *Eur J Clin Invest,* 38 Suppl 2, 2-11.
DHO-MOULIN, M. & FAIRBROTHER, J. M. (1999) Avian pathogenic *Escherichia coli* (APEC). *Vet Res,* 30, 299-316.
DOBRINDT, U. (2005) (Patho-)Genomics of *Escherichia coli*. *Int J Med Microbiol,* 295, 357-71.
DOBRINDT, U. & HACKER, J. (2008) Targeting virulence traits: potential strategies to combat extraintestinal pathogenic *Escherichia coli* infections. *Curr Opin Microbiol,* 11, 409-13.
DONG, T. & SCHELLHORN, H. E. (2009a) Control of RpoS in global gene expression of *Escherichia coli* in minimal media. *Mol Genet Genomics,* 281, 19-33.
DONG, T. & SCHELLHORN, H. E. (2009b) Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933. *BMC Genomics,* 10, 349.
DONNENBERG, M. S. & KAPER, J. B. (1992) Enteropathogenic *Escherichia coli*. *Infect Immun,* 60, 3953-61.
DORN, C. R. & ANGRICK, E. J. (1991) Serotype O157:H7 *Escherichia coli* from bovine and meat sources. *J Clin Microbiol,* 29, 1225-31.
DOZOIS, C. M., DHO-MOULIN, M., BREE, A., FAIRBROTHER, J. M., DESAUTELS, C. & CURTISS, R. (2000) Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. *Infect Immun,* 68, 4145-4154.
DUNDAS, S., MURPHY, J., SOUTAR, R. L., JONES, G. A., HUTCHINSON, S. J. & TODD, W. T. (1999) Effectiveness of therapeutic plasma exchange in the 1996 Lanarkshire *Escherichia coli* O157:H7 outbreak. *Lancet,* 354, 1327-30.
DUPONT, H. L. (2009) Clinical practice. Bacterial diarrhea. *N Engl J Med,* 361, 1560-9.
DUROCHER, D. & JACKSON, S. P. (2002) The FHA domain. *FEBS Lett,* 513, 58-66.
DUROCHER, D., TAYLOR, I. A., SARBASSOVA, D., HAIRE, L. F., WESTCOTT, S. L., JACKSON, S. P., SMERDON, S. J. & YAFFE, M. B. (2000) The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. *Mol Cell,* 6, 1169-82.
DURSO, L. M., REYNOLDS, K., BAUER, N., JR. & KEEN, J. E. (2005) Shiga-toxigenic *Escherichia coli* O157:H7 infections among livestock exhibitors and visitors at a Texas County Fair. *Vector Borne Zoonotic Dis,* 5, 193-201.
DUTTON, R. J., XU, Z. & GOBER, J. W. (2005) Linking structural assembly to gene expression: a novel mechanism for regulating the activity of a sigma54 transcription factor. *Mol Microbiol,* 58, 743-57.
DZIVA, F., VAN DIEMEN, P. M., STEVENS, M. P., SMITH, A. J. & WALLIS, T. S. (2004) Identification of *Escherichia coli* O157:H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. *Microbiology,* 150, 3631-45.
EBEL, F., DEIBEL, C., KRESSE, A. U., GUZMAN, C. A. & CHAKRABORTY, T. (1996) Temperature- and medium-dependent secretion of

proteins by Shiga toxin-producing *Escherichia coli*. *Infect Immun,* 64, 4472-4479.
EBEL, F., PODZADEL, T., ROHDE, M., KRESSE, A. U., KRAMER, S., DEIBEL, C., GUZMAN, C. A. & CHAKRABORTY, T. (1998) Initial binding of Shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. *Mol Microbiol,* 30, 147-161.
EDQVIST, P. J., BROMS, J. E., BETTS, H. J., FORSBERG, A., PALLEN, M. J. & FRANCIS, M. S. (2006) Tetratricopeptide repeats in the type III secretion chaperone, LcrH: their role in substrate binding and secretion. *Mol Microbiol,* 59, 31-44.
EDWARDS, R. A. & SCHIFFERLI, D. M. (1997) Differential regulation of fasA and fasH expression of *Escherichia coli* 987P fimbriae by environmental cues. *Mol Microbiol,* 25, 797-809.
EICHELBERG, K. & GALAN, J. E. (2000) The flagellar sigma factor FliA (σ^{28}) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. *Infect Immun,* 68.
ELDER, R. O., KEEN, J. E., SIRAGUSA, G. R., BARKOCY-GALLAGHER, G. A., KOOHMARAIE, M. & LAEGREID, W. W. (2000) Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci U S A,* 97, 2999-3003.
ELLIOTT, S. J., HUTCHESON, S. W., DUBOIS, M. S., MELLIES, J. L., WAINWRIGHT, L. A., BATCHELOR, M., FRANKEL, G., KNUTTON, S. & KAPER, J. B. (1999a) Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol,* 33, 1176-1189.
ELLIOTT, S. J., KREJANY, E. O., MELLIES, J. L., ROBINS-BROWNE, R. M., SASAKAWA, C. & KAPER, J. B. (2001) EspG, a novel type III system-secreted protein from enteropathogenic *Escherichia coli* with similarities to VirA of *Shigella flexneri*. *Infect Immun,* 69, 4027-33.
ELLIOTT, S. J., O'CONNELL, C. B., KOUTSOURIS, A., BRINKLEY, C., DONNENBERG, M. S., HECHT, G. & KAPER, J. B. (2002) A gene from the locus of enterocyte effacement that is required for enteropathogenic *Escherichia coli* to increase tight-junction permeability encodes a chaperone for EspF. *Infect Immun,* 70, 2271-7.
ELLIOTT, S. J., SPERANDIO, V., GIRON, J. A., SHIN, S., MELLIES, J. L., WAINWRIGHT, L., HUTCHESON, S. W., MCDANIEL, T. K. & KAPER, J. B. (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun,* 68, 6115-26.
ELLIOTT, S. J., YU, J. & KAPER, J. B. (1999b) The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *Escherichia coli* K-12. *Infect Immun,* 67, 4260-3.
EMMERSON, J. R., GALLY, D. L. & ROE, A. J. (2006) Generation of gene deletions and gene replacements in *Escherichia coli* O157:H7 using a temperature sensitive allelic exchange system. *Biol Proced Online,* 8, 153-62.

ERHARDT, M., HIRANO, T., SU, Y., PAUL, K., WEE, D. H., MIZUNO, S., AIZAWA, S. & HUGHES, K. T. (2010) The role of the FliK molecular ruler in hook-length control in *Salmonella enterica*. *Mol Microbiol,* 75, 1272-84.
ESCHERICH, T. (1885) Die darmbakterien des neugeborenen und sauglings. *Fortshr. Med.* , 3:5-15-522, 547-554.
EVANS, L. D., STAFFORD, G. P., AHMED, S., FRASER, G. M. & HUGHES, C. (2006) An escort mechanism for cycling of export chaperones during flagellum assembly. *Proc Natl Acad Sci U S A,* 103, 17474-9.
FERRACCI, F., DAY, J. B., EZELLE, H. J. & PLANO, G. V. (2004) Expression of a functional secreted YopN-TyeA hybrid protein in *Yersinia pestis* is the result of a +1 translational frameshift event. *J Bacteriol,* 186, 5160-6.
FERRACCI, F., SCHUBOT, F. D., WAUGH, D. S. & PLANO, G. V. (2005) Selection and characterization of *Yersinia pestis* YopN mutants that constitutively block Yop secretion. *Mol Microbiol,* 57, 970-87.
FERRIS, H. U., FURUKAWA, Y., MINAMINO, T., KROETZ, M. B., KIHARA, M., NAMBA, K. & MACNAB, R. M. (2005) FlhB regulates ordered export of flagellar components via autocleavage mechanism. *J Biol Chem,* 280, 41236-42.
FILLOUX, A., HACHANI, A. & BLEVES, S. (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology,* 154, 1570-83.
FLECKENSTEIN, J. M., HARDWIDGE, P. R., MUNSON, G. P., RASKO, D. A., SOMMERFELT, H. & STEINSLAND, H. (2010) Molecular mechanisms of enterotoxigenic *Escherichia coli* infection. *Microbes Infect,* 12, 89-98.
FOLICHON, M., ARLUISON, V., PELLEGRINI, O., HUNTZINGER, E., REGNIER, P. & HAJNSDORF, E. (2003) The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res,* 31, 7302-10.
FORSBERG, A., VIITANEN, A. M., SKURNIK, M. & WOLF-WATZ, H. (1991) The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol Microbiol,* 5, 977-86.
FRANKEL, G., PHILLIPS, A. D., NOVAKOVA, M., FIELD, H., CANDY, D. C., SCHAUER, D. B., DOUCE, G. & DOUGAN, G. (1996) Intimin from enteropathogenic *Escherichia coli* restores murine virulence to a *Citrobacter rodentium* eaeA mutant: induction of an immunoglobulin A response to intimin and EspB. *Infect Immun,* 64, 5315-25.
FRANKEL, G., PHILLIPS, A. D., ROSENSHINE, I., DOUGAN, G., KAPER, J. B. & KNUTTON, S. (1998) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol,* 30, 911-921.
FRANZE DE FERNANDEZ, M. T., EOYANG, L. & AUGUST, J. T. (1968) Factor fraction required for the synthesis of bacteriophage Qbeta-RNA. *Nature,* 219, 588-90.
FRASER, G. M., BENNETT, J. C. & HUGHES, C. (1999) Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. *Mol Microbiol,* 32, 569-80.
FREEMAN, J. A., RAPPL, C., KUHLE, V., HENSEL, M. & MILLER, S. I. (2002) SpiC is required for translocation of *Salmonella* pathogenicity island 2

effectors and secretion of translocon proteins SseB and SseC. *J Bacteriol,* 184, 4971-80.
FRIEBEL, A., ILCHMANN, H., AELPFELBACHER, M., EHRBAR, K., MACHLEIDT, W. & HARDT, W. D. (2001) SopE and SopE2 from *Salmonella typhimurium* activate different sets of Rho GTPases of the host cell. *J Biol Chem,* 276, 34035-34040.
FRIEDBERG, D., UMANSKI, T., FANG, Y. & ROSENSHINE, I. (1999) Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Mol Microbiol,* 34, 941-52.
FUKIYA, S., MIZOGUCHI, H., TOBE, T. & MORI, H. (2004) Extensive genomic diversity in pathogenic *Escherichia coli* and *Shigella* Strains revealed by comparative genomic hybridization microarray. *J Bacteriol,* 186, 3911-21.
GALAN, J. E. (2008) Energizing type III secretion machines: what is the fuel? *Nat Struct Mol Biol,* 15, 127-8.
GALAN, J. E. & WOLF-WATZ, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature,* 444, 567-73.
GALPERIN, M., DIBROV, P. A. & GLAGOLEV, A. N. (1982) delta mu H+ is required for flagellar growth in *Escherichia coli*. *FEBS Lett,* 143, 319-22.
GAMAGE, S. D., PATTON, A. K., STRASSER, J. E., CHALK, C. L. & WEISS, A. A. (2006) Commensal bacteria influence *Escherichia coli* O157:H7 persistence and Shiga toxin production in the mouse intestine. *Infect Immun,* 74, 1977-83.
GARCIA-ANGULO, V. A., DENG, W., THOMAS, N. A., FINLAY, B. B. & PUENTE, J. L. (2008) Regulation of expression and secretion of NleH, a new non-locus of enterocyte effacement-encoded effector in *Citrobacter rodentium*. *J Bacteriol,* 190, 2388-99.
GARMENDIA, J., PHILLIPS, A. D., CARLIER, M. F., CHONG, Y., SCHULLER, S., MARCHES, O., DAHAN, S., OSWALD, E., SHAW, R. K., KNUTTON, S. & FRANKEL, G. (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol,* 6, 1167-83.
GAUTHIER, A. & FINLAY, B. B. (2003) Translocated intimin receptor and its chaperone interact with ATPase of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *J Bacteriol,* 185, 6747-55.
GAUTHIER, A., PUENTE, J. L. & FINLAY, B. B. (2003a) Secretin of the enteropathogenic *Escherichia coli* type III secretion system requires components of the type III apparatus for assembly and localization. *Infect Immun,* 71, 3310-9.
GAUTHIER, A., THOMAS, N. A. & FINLAY, B. B. (2003b) Bacterial injection machines. *J Biol Chem,* 278, 25273-6.
GENIN, S., GOUGH, C. L., ZISCHEK, C. & BOUCHER, C. A. (1992) Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol Microbiol,* 6, 3065-76.
GINOCCHIO, C. C., OLMSTED, S. B., WELLS, C. L. & GALAN, J. E. (1994) Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. *Cell,* 76, 717-24.
GIRON, J. A., TORRES, A. G., FREER, E. & KAPER, J. B. (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol,* 44, 361-379.

GOLDBERG, M. D., JOHNSON, M., HINTON, J. C. & WILLIAMS, P. H. (2001) Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. *Mol Microbiol,* 41, 549-59.
GOMEZ-DUARTE, O. G. & KAPER, J. B. (1995) A plasmid-encoded regulatory region activates chromosomal eaeA expression in enteropathogenic *Escherichia coli*. *Infect Immun,* 63, 1767-76.
GONZALEZ-PEDRAJO, B., MINAMINO, T., KIHARA, M. & NAMBA, K. (2006) Interactions between C ring proteins and export apparatus components: a possible mechanism for facilitating type III protein export. *Mol Microbiol,* 60, 984-98.
GOODE, B., O'REILLY, C., DUNN, J., FULLERTON, K., SMITH, S., GHNEIM, G., KEEN, J., DURSO, L., DAVIES, M. & MONTGOMERY, S. (2009) Outbreak of *Escherichia coli* O157: H7 infections after Petting Zoo visits, North Carolina State Fair, October-November 2004. *Arch Pediatr Adolesc Med,* 163, 42-8.
GOPHNA, U., RON, E. Z. & GRAUR, D. (2003) Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. *Gene,* 312, 151-63.
GOUGH, C. L., GENIN, S., ZISCHEK, C. & BOUCHER, C. A. (1992) *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol Plant Microbe Interact,* 5, 384-9.
GRIFFIN, P. M., OSTROFF, S. M., TAUXE, R. V., GREENE, K. D., WELLS, J. G., LEWIS, J. H. & BLAKE, P. A. (1988) Illnesses associated with *Escherichia coli* O157:H7 infections. A broad clinical spectrum. *Ann Intern Med,* 109, 705-12.
GRUENHEID, S., SEKIROV, I., THOMAS, N. A., DENG, W., O'DONNELL, P., GOODE, D., LI, Y., FREY, E. A., BROWN, N. F., METALNIKOV, P., PAWSON, T., ASHMAN, K. & FINLAY, B. B. (2004) Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol,* 51, 1233-49.
HAACK, K. R., ROBINSON, C. L., MILLER, K. J., FOWLKES, J. W. & MELLIES, J. L. (2003) Interaction of Ler at the LEE5 (*tir*) operon of enteropathogenic *Escherichia coli*. *Infect Immun,* 71, 384-92.
HACKER, J., KESTLER, H., HOSCHUTZKY, H., JANN, K., LOTTSPEICH, F. & KORHONEN, T. K. (1993) Cloning and characterization of the S-fimbrial adhesin II complex of an *Escherichia coli* O18:K1 Meningitis Isolate. *Infect Immun,* 61, 544-550.
HAKANSSON, S., BERGMAN, T., VANOOTEGHEM, J. C., CORNELIS, G. & WOLF-WATZ, H. (1993) YopB and YopD constitute a novel class of *Yersinia* Yop proteins. *Infect Immun,* 61, 71-80.
HANSEN, A. M. & KAPER, J. B. (2009) Hfq affects the expression of the LEE pathogenicity island in enterohaemorrhagic *Escherichia coli*. *Mol Microbiol,* 73, 446-65.
HARTL, D. L. & DYKHUIZEN, D. E. (1984) The population genetics of *Escherichia coli*. *Annu Rev Genet,* 18, 31-68.
HARTLAND, E. L., BATCHELOR, M., DELAHAY, R. M., HALE, C., MATTHEWS, S., DOUGAN, G., KNUTTON, S., CONNERTON, I. &

FRANKEL, G. (1999) Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Mol Microbiol,* 32, 151-158.
HARTLAND, E. L., BORDUN, A. M. & ROBINS-BROWNE, R. M. (1996) Contribution of YopB to virulence of *Yersinia enterocolitica*. *Infect Immun,* 64, 2308-14.
HASHIMOTO, H., MIZUKOSHI, K., NISHI, M., KAWAKITA, T., HASUI, S., KATO, Y., UENO, Y., TAKEYA, R., OKUDA, N. & TAKEDA, T. (1999) Epidemic of gastrointestinal tract infection including hemorrhagic colitis attributable to Shiga toxin 1-producing *Escherichia coli* O118:H2 at a junior high school in Japan. *Pediatrics,* 103, E21-E25.
HEDBERG, C. W., SAVARINO, S. J., BESSER, J. M., PAULUS, C. J., THELEN, V. M., MYERS, L. J., CAMERON, D. N., BARRETT, T. J., KAPER, J. B., OSTERHOLM, M. T., BOYER, W., KAIRIS, F., GABRIEL, L., SOLER, J., GYSWYT, L., BRAY, S., CARLSON, R., HOOKER, C., FASANO, A., JARVIS, K., MCDANIEL, T. & TORNIEPORTH, N. (1997) An outbreak of foodborne illness caused by *Escherichia coli* O39:NM, an agent not fitting into the existing scheme for classifying diarrheogenic *E. coli*. *J Infect Dis,* 176, 1625-1628.
HENDERSON, I. R., NAVARRO-GARCIA, F., DESVAUX, M., FERNANDEZ, R. C. & ALA'ALDEEN, D. (2004) Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev,* 68, 692-744.
HEUVELINK, A. E., VANDENBIGGELAAR, F. L. A. M., ZWARTKRUISNAHUIS, J. T. M., HERBES, R. G., HUYBEN, R., NAGELKERKE, N., MELCHERS, W. J. G., MONNENS, L. A. H. & DEBOER, E. (1998) Occurrence of verocytotoxin-producing *Escherichia coli* O157 on Dutch daily farms. *J Clin Microbiol,* 36, 3480-3487.
HICKS, S., FRANKEL, G., KAPER, J. B., DOUGAN, G. & PHILLIPS, A. D. (1998) Role of intimin and bundle-forming pili in enteropathogenic *Escherichia coli* adhesion to pediatric intestinal tissue *in vitro*. *Infect Immun,* 66, 1570-1578.
HIGASHIDE, W. & ZHOU, D. (2006) The first 45 amino acids of SopA are necessary for InvB binding and SPI-1 secretion. *J Bacteriol,* 188, 2411-20.
HILL, S. M., PHILLIPS, A. D. & WALKER-SMITH, J. A. (1991) Enteropathogenic *Escherichia coli* and life threatening chronic diarrhoea. *Gut,* 32, 154-8.
HOBSON, N., PRICE, N. L., WARD, J. D. & RAIVIO, T. L. (2008) Generation of a restriction minus enteropathogenic *Escherichia coli* E2348/69 strain that is efficiently transformed with large, low copy plasmids. *BMC Microbiol,* 8, 134.
HOFMANN, K. & BUCHER, P. (1995) The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. *Trends Biochem Sci,* 20, 347-9.
HOLLAND, I. B., SCHMITT, L. & YOUNG, J. (2005) Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). *Mol Membr Biol,* 22, 29-39.
HOMMAIS, F., KRIN, E., LAURENT-WINTER, C., SOUTOURINA, O., MALPERTUY, A., LE CAER, J. P., DANCHIN, A. & BERTIN, P. (2001) Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. *Mol Microbiol,* 40, 20-36.

HU, X., LEE, M. S. & WALLQVIST, A. (2009) Interaction of the disordered *Yersinia* effector protein YopE with its cognate chaperone SycE. *Biochemistry,* 48, 11158-60.
HUANG, L. H. & SYU, W. J. (2008) GrlA of enterohemorrhagic *Escherichia coli* O157:H7 activates LEE1 by binding to the promoter region. *J Microbiol Immunol Infect,* 41, 9-16.
HUECK, C. J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev,* 62, 379-433.
HUPPERTZ, H. I., BUSCH, D., SCHMIDT, H., ALEKSIC, S. & KARCH, H. (1996) Diarrhea in young children associated with *Escherichia coli* non-O157 organisms that produce Shiga-like toxin. *J Pediatr,* 128, 341-346.
IDE, T., LAARMANN, S., GREUNE, L., SCHILLERS, H., OBERLEITHNER, H. & SCHMIDT, M. A. (2001) Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cell Microbiol,* 3, 669-79.
IDE, T., MICHGEHL, S., KNAPPSTEIN, S., HEUSIPP, G. & SCHMIDT, M. A. (2003) Differential modulation by Ca^{2+} of type III secretion of diffusely adhering enteropathogenic *Escherichia coli*. *Infect Immun,* 71, 1725-32.
IRIARTE, M., SORY, M. P., BOLAND, A., BOYD, A. P., MILLS, S. D., LAMBERMONT, I. & CORNELIS, G. R. (1998) TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *EMBO J,* 17, 1907-18.
IYODA, S. & WATANABE, H. (2004) Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157 : H7 to HEp-2 cells. *Microbiology,* 150, 2357-571.
JACKSON, D. W., SUZUKI, K., OAKFORD, L., SIMECKA, J. W., HART, M. E. & ROMEO, T. (2002) Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J Bacteriol,* 184, 290-301.
JACKSON, L. A., KEENE, W. E., MCANULTY, J. M., ALEXANDER, E. R., DIERMAYER, M., DAVIS, M. A., HEDBERG, K., BOASE, J., BARRETT, T. J., SAMADPOUR, M. & FLEMING, D. W. (2000) Where's the beef? The role of cross-contamination in 4 chain restaurant-associated outbreaks of *Escherichia coli* O157:H7 in the Pacific Northwest. *Arch Intern Med,* 160, 2380-5.
JACKSON, M. W., DAY, J. B. & PLANO, G. V. (1998) YscB of *Yersinia pestis* functions as a specific chaperone for YopN. *J Bacteriol,* 180, 4912-21.
JARVIS, K. G., GIRON, J. A., JERSE, A. E., MCDANIEL, T. K., DONNENBERG, M. S. & KAPER, J. B. (1995) Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci U S A,* 92, 7996-8000.
JARVIS, K. G. & KAPER, J. B. (1996) Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secretion system. *Infect Immun,* 64, 4826-9.
JAY, M. T., COOLEY, M., CARYCHAO, D., WISCOMB, G. W., SWEITZER, R. A., CRAWFORD-MIKSZA, L., FARRAR, J. A., LAU, D. K., O'CONNELL, J., MILLINGTON, A., ASMUNDSON, R. V., ATWILL, E. R. & MANDRELL, R. E. (2007) *Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast. *Emerg Infect Dis,* 13, 1908-11.

JOBICHEN, C., LI, M., YERUSHALMI, G., TAN, Y. W., MOK, Y. K., ROSENSHINE, I., LEUNG, K. Y. & SIVARAMAN, J. (2007) Structure of GrlR and the implication of its EDED motif in mediating the regulation of type III secretion system in EHEC. *PLoS Pathog,* 3, e69.
JOHNSON, J. R. (1991) Virulence factors in *Escherichia coli* urinary tract infection. *Clin Microbiol Rev,* 4, 80-128.
JOHNSON, J. R., MURRAY, A. C., GAJEWSKI, A., SULLIVAN, M., SNIPPES, P., KUSKOWSKI, M. A. & SMITH, K. E. (2003) Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products. *Antimicrob Agents Chemother,* 47, 2161-8.
JOHNSON, J. R. & RUSSO, T. A. (2002) Extraintestinal pathogenic *Escherichia coli*: "the other bad E coli". *J Lab Clin Med,* 139, 155-62.
JOHNSON, J. R. & RUSSO, T. A. (2005) Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli*. *Int J Med Microbiol,* 295, 383-404.
JOHNSON, S., ROVERSI, P., ESPINA, M., OLIVE, A., DEANE, J. E., BIRKET, S., FIELD, T., PICKING, W. D., BLOCKER, A. J., GALYOV, E. E., PICKING, W. L. & LEA, S. M. (2007) Self-chaperoning of the type III secretion system needle tip proteins IpaD and BipD. *J Biol Chem,* 282, 4035-44.
JOHNSON, T. J., JORDAN, D., KARIYAWASAM, S., STELL, A. L., BELL, N. P., WANNEMUEHLER, Y. M., ALARCON, C. F., LI, G., TIVENDALE, K. A., LOGUE, C. M. & NOLAN, L. K. (2010) Sequence analysis and characterization of a transferable hybrid plasmid encoding multidrug resistance and enabling zoonotic potential for extraintestinal *Escherichia coli*. *Infect Immun,* 78, 1931-42.
JOHNSON, T. J. & NOLAN, L. K. (2009) Pathogenomics of the virulence plasmids of *Escherichia coli*. *Microbiol Mol Biol Rev,* 73, 750-74.
JORES, J., ZEHMKE, K., EICHBERG, J., RUMER, L. & WIELER, L. H. (2003) Description of a novel intimin variant (type zeta) in the bovine O84:NM verotoxin-producing *Escherichia coli* strain 537/89 and the diagnostic value of intimin typing. *Exp Biol Med (Maywood),* 228, 370-6.
JOURNET, L., AGRAIN, C., BROZ, P. & CORNELIS, G. R. (2003) The needle length of bacterial injectisomes is determined by a molecular ruler. *Science,* 302, 1757-1760.
JOURNET, L., HUGHES, K. T. & CORNELIS, G. R. (2005) Type III secretion: a secretory pathway serving both motility and virulence. *Mol Membr Biol,* 22, 41-50.
JUAREZ, A., NIETO, J. M., PRENAFETA, A., MIQUELAY, E., BALSALOBRE, C., CARRASCAL, M. & MADRID, C. (2000) Interaction of the nucleoid-associated proteins Hha and H-NS to modulate expression of the hemolysin operon in *Escherichia coli*. *Adv Exp Med Biol,* 485, 127-31.
JUSTICE, S. S., LAUER, S. R., HULTGREN, S. J. & HUNSTAD, D. A. (2006) Maturation of intracellular *Escherichia coli* communities requires SurA. *Infect Immun,* 74, 4793-800.
KANACK, K. J., CRAWFORD, J. A., TATSUNO, I., KARMALI, M. A. & KAPER, J. B. (2005) SepZ/EspZ is secreted and translocated into HeLa cells by the enteropathogenic *Escherichia coli* type III secretion system. *Infect Immun,* 73, 4327-37.

KAPER, J. B., MCDANIEL, T. K., JARVIS, K. G. & GOMEZDUARTE, O. (1997) Genetics of virulence of enteropathogenic *E. coli*. *Adv Exp Med Biol,* 412, 279-287.
KAPER, J. B., NATARO, J. P. & MOBLEY, H. L. (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol,* 2, 123-40.
KARAOLIS, D. K. R., MCDANIEL, T. K., KAPER, J. B. & BOEDEKER, E. C. (1997) Cloning of the RDEC-1 locus of enterocyte effacement (LEE) and functional analysis of the phenotype on HEP-2 cells. *Adv Exp Med Biol,* 412, 241-245.
KARAVOLOS, M. H., ROE, A. J., WILSON, M., HENDERSON, J., LEE, J. J., GALLY, D. L. & KHAN, C. M. (2005) Type III secretion of the *Salmonella* effector protein SopE is mediated via an N-terminal amino acid signal and not an mRNA sequence. *J Bacteriol,* 187, 1559-67.
KARCH, H., BOHM, H., SCHMIDT, H., GUNZER, F., ALEKSIC, S. & HEESEMANN, J. (1993) Clonal structure and pathogenicity of Shiga-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H. *J Clin Microbiol,* 31, 1200-5.
KARLINSEY, J. E., LONNER, J., BROWN, K. L. & HUGHES, K. T. (2000a) Translation/secretion coupling by type III secretion systems. *Cell,* 102, 487-97.
KARLINSEY, J. E., TANAKA, S., BETTENWORTH, V., YAMAGUCHI, S., BOOS, W., AIZAWA, S. I. & HUGHES, K. T. (2000b) Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and fliC transcription. *Mol Microbiol,* 37, 1220-31.
KARLINSEY, J. E., TSUI, H. C., WINKLER, M. E. & HUGHES, K. T. (1998) Flk couples flgM translation to flagellar ring assembly in *Salmonella typhimurium*. *J Bacteriol,* 180, 5384-97.
KARMALI, M., PETRIC, M., LIM, C., FLEMING, P. & STEELE, B. (1983) *Escherichia coli* cytotoxin, hemolytic uremic syndrome and hemorrhagic colitis. *Lancet,* 2, 1299-1300.
KAUFFMANN, F. (1944) ZürSerologie der Coli-Gruppe. *Acta path. microbiol. scand.,* 21, 20-45.
KAUFFMANN, F. (1947) The serology of the coli group. *J. Immunol.,* 57, 71.
KEEN, J. E., T. E. WITTUM, J. R. DUNN, J. L. BONO, AND M. E. FONTENOT. (2003) Occurrence of STEC O157, O111, and O26 in livestock at agricultural fairs in the United States. . *Page 22 in Proc. 5th Int. Symp. on Shiga Toxin-Producing Escherichia coli Infections, Edinburgh, U.K.* .
KEENAN, K. P., SHARPNACK, D. D., COLLINS, H., FORMAL, S. B. & O'BRIEN, A. D. (1986) Morphologic evaluation of the effects of Shiga toxin and E coli Shiga-like toxin on the rabbit intestine. *Am J Pathol,* 125, 69-80.
KELLY, M., HART, E., MUNDY, R., MARCHES, O., WILES, S., BADEA, L., LUCK, S., TAUSCHEK, M., FRANKEL, G., ROBINS-BROWNE, R. M. & HARTLAND, E. L. (2006) Essential role of the type III secretion system effector NleB in colonization of mice by *Citrobacter rodentium*. *Infect Immun,* 74, 2328-2337.
KENNY, B. (1999) Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. *Mol Microbiol,* 31, 1229-41.

KENNY, B., ABE, A., STEIN, M. & FINLAY, B. B. (1997a) Enteropathogenic *Escherichia coli* protein secretion is induced in response to conditions similar to those in the gastrointestinal tract. *Infect Immun,* 65, 2606-2612.
KENNY, B., DEVINNEY, R., STEIN, M., REINSCHEID, D. J., FREY, E. A. & FINLAY, B. B. (1997b) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell,* 91, 511-520.
KENNY, B., ELLIS, S., LEARD, A. D., WARAWA, J., MELLOR, H. & JEPSON, M. A. (2002) Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. *Mol Microbiol,* 44, 1095-1107.
KENNY, B. & FINLAY, B. B. (1997) Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-gamma 1. *Infect Immun,* 65, 2528-2536.
KIM, D. W., LENZEN, G., PAGE, A. L., LEGRAIN, P., SANSONETTI, P. J. & PARSOT, C. (2005) The *Shigella flexneri* effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proc Natl Acad Sci U S A,* 102, 14046-14051.
KIM, J., THANABALASURIAR, A., CHAWORTH-MUSTERS, T., FROMME, J. C., FREY, E. A., LARIO, P. I., METALNIKOV, P., RIZG, K., THOMAS, N. A., LEE, S. F., HARTLAND, E. L., HARDWIDGE, P. R., PAWSON, T., STRYNADKA, N. C., FINLAY, B. B., SCHEKMAN, R. & GRUENHEID, S. (2007) The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. *Cell Host Microbe,* 2, 160-71.
KIM, K. S. (2002) Strategy of *Escherichia coli* for crossing the blood-brain barrier. *J Infect Dis,* 186 Suppl 2, S220-4.
KLAPPROTH, J. M. A., SASAKI, M., SHERMAN, M., BABBIN, B., DONNENBERG, M. S., FERNANDES, P. J., SCALETSKY, I. C. A., KALMAN, D., NUSRAT, A. & WILLIAMS, I. R. (2005) *Citrobacter rodentium* lifA/efa1 is essential for colonic colonization and crypt cell hyperplasia *in vivo*. *Infect Immun,* 73, 1441-1451.
KLINE, K. A., DODSON, K. W., CAPARON, M. G. & HULTGREN, S. J. A tale of two pili: assembly and function of pili in bacteria. *Trends Microbiol,* 18, 224-32.
KNODLER, L. A., CELLI, J., HARDT, W. D., VALLANCE, B. A., YIP, C. & FINLAY, B. B. (2002) *Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. *Mol Microbiol,* 43, 1089-1103.
KNUTTON, S., BALDWIN, T., WILLIAMS, P. H. & MCNEISH, A. S. (1989a) Actin accumulation at sites of bacterial adhesion to tissue culture cells - basis of a new diagnostic test for Enteropathogenic and Enterohemorrhagic *Escherichia coli*. *Infect Immun,* 57, 1290-1298.
KNUTTON, S., BALDWIN, T., WILLIAMS, P. H. & MCNEISH, A. S. (1989b) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun,* 57, 1290-8.
KNUTTON, S., ROSENSHINE, I., PALLEN, M. J., NISAN, I., NEVES, B. C., BAIN, C., WOLFF, C., DOUGAN, G. & FRANKEL, G. (1998) A novel EspA-

associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J,* 17, 2166-2176.
KNUTTON, S., SHAW, R., MCNEISH, A. S., PHILIPS, A., PRICE, E. & WATSON, P. (1989c) Diagnosis of enteropathogenic *Escherichia coli*. *Lancet,* 2, 218.
KONKEL, M. E., KLENA, J. D., RIVERA-AMILL, V., MONTEVILLE, M. R., BISWAS, D., RAPHAEL, B. & MICKELSON, J. (2004) Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol,* 186, 3296-303.
KOUTKIA, P., MYLONAKIS, E. & FLANIGAN, T. (1997) Enterohemorrhagic *Escherichia coli* O157:H7 - An emerging pathogen. *Am Fam Physician,* 56, 853-856.
KRESSE, A. U., BELTRAMETTI, F., MULLER, A., EBEL, F. & GUZMAN, C. A. (2000) Characterization of SepL of enterohemorrhagic *Escherichia coli*. *J Bacteriol,* 182, 6490-8.
KRESSE, A. U., SCHULZE, K., DEIBEL, C., EBEL, F., ROHDE, M., CHAKRABORTY, T. & GUZMAN, C. A. (1998) Pas, a novel protein required for protein secretion and attaching and effacing activities of enterohemorrhagic *Escherichia coli*. *J Bacteriol,* 180, 4370-9.
KROHN, M. A., THWIN, S. S., RABE, L. K., BROWN, Z. & HILLIER, S. L. (1997) Vaginal colonization by *Escherichia coli* as a risk factor for very low birth weight delivery and other perinatal complications. *J Infect Dis,* 175, 606-10.
KUBORI, T. & GALAN, J. E. (2002) *Salmonella* type III secretion-associated protein InvE controls translocation of effector proteins into host cells. *J Bacteriol,* 184, 4699-708.
LAABERKI, M. H., JANABI, N., OSWALD, E. & REPOILA, F. (2006) Concert of regulators to switch on LEE expression in enterohemorrhagic *Escherichia coli* O157:H7: interplay between Ler, GrlA, HNS and RpoS. *Int J Med Microbiol,* 296, 197-210.
LAN, R., ALLES, M. C., DONOHOE, K., MARTINEZ, M. B. & REEVES, P. R. (2004) Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. *Infect Immun,* 72, 5080-8.
LAN, R. & REEVES, P. R. (2002) *Escherichia coli* in disguise: molecular origins of *Shigella*. *Microbes Infect,* 4, 1125-32.
LANE, M. C. & MOBLEY, H. L. (2007) Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic *Escherichia coli* (UPEC) in the mammalian kidney. *Kidney Int,* 72, 19-25.
LANE, M. C., SIMMS, A. N. & MOBLEY, H. L. (2007) complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic *Escherichia coli*. *J Bacteriol,* 189, 5523-33.
LAVANDER, M., SUNDBERG, L., EDQVIST, P. J., LLOYD, S. A., WOLF-WATZ, H. & FORSBERG, A. (2002) Proteolytic cleavage of the FlhB homologue YscU of *Yersinia pseudotuberculosis* is essential for bacterial survival but not for type III secretion. *J Bacteriol,* 184, 4500-9.
LAW, D. (1994) Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. *Clin Microbiol Rev,* 7, 152-73.
LEASE, R. A. & BELFORT, M. (2000) Riboregulation by DsrA RNA: trans-actions for global economy. *Mol Microbiol,* 38, 667-72.

LEATHAM, M. P., BANERJEE, S., AUTIERI, S. M., MERCADO-LUBO, R., CONWAY, T. & COHEN, P. S. (2009) Precolonized human commensal *Escherichia coli* strains serve as a barrier to *Escherichia coli* O157:H7 growth in the streptomycin-treated mouse intestine. *Infect Immun,* 77, 2876-86.
LEDERBERG, J. (1996) Genetic recombination in *Escherichia coli*: disputation at Cold Spring Harbor, 1946-1996. *Genetics,* 144, 439-43.
LEDERBERG, J. Tatum, E. L.. (1946) Gene recombination in *Escherichia coli*. *Nature*, 158, 558.
LEE, H. J. & HUGHES, K. T. (2006) Posttranscriptional control of the *Salmonella enterica* flagellar hook protein FlgE. *J Bacteriol,* 188, 3308-16.
LEE, S. H. & GALAN, J. E. (2004) *Salmonella* type III secretion-associated chaperones confer secretion-pathway specificity. *Mol Microbiol,* 51, 483-495.
LEE, S. Y. (1996) High cell-density culture of *Escherichia coli*. *Trends Biotechnol,* 14, 98-105.
LEE, S. Y., COSTELLO, M. & KANG, D. H. (2004) Efficacy of chlorine dioxide gas as a sanitizer of lettuce leaves. *J Food Prot,* 67, 1371-6.
LETZELTER, M., SORG, I., MOTA, L. J., MEYER, S., STALDER, J., FELDMAN, M., KUHN, M., CALLEBAUT, I. & CORNELIS, G. R. (2006) The discovery of SycO highlights a new function for type III secretion effector chaperones. *EMBO J,* 25, 3223-33.
LEVINE, M. M. (1984) *Escherichia coli* infections. IN GERMANIER, R. (Ed.) *Bacterial Vaccines.* New York: Academic.
LEVINE, M. M. (1987) *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J Infect Dis,* 155, 377-89.
LEVINE, M. M., BERGQUIST, E. J., NALIN, D. R., WATERMAN, D. H., HORNICK, R. B., YOUNG, C. R. & SOTMAN, S. (1978) *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet,* 1, 1119-22.
LEVINE, M. M. & EDELMAN, R. (1984) Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. *Epidemiol Rev,* 6, 31-51.
LI, M., ROSENSHINE, I., YU, H. B., NADLER, C., MILLS, E., HEW, C. L. & LEUNG, K. Y. (2006) Identification and characterization of NleI, a new non-LEE-encoded effector of enteropathogenic *Escherichia coli* (EPEC). *Microbes Infect,* 8, 2890-8.
LINGWOOD, C. A. (1993) Verotoxins and their glycolipid receptors. *Adv Lipid Res,* 25, 189-211.
LINGWOOD, C. A., LAW, H., RICHARDSON, S., PETRIC, M., BRUNTON, J. L., DEGRANDIS, S. & KARMALI, M. (1987) Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *In vitro*. *J Biol Chem,* 262, 8834-8839.
LIO, J. C. & SYU, W. J. (2004) Identification of a negative regulator for the pathogenicity island of enterohemorrhagic *Escherichia coli* O157:H7. *J Biomed Sci,* 11, 855-63.
LITERAK, I., DOLEJSKA, M., RYBARIKOVA, J., CIZEK, A., STREJCKOVA, P., VYSKOCILOVA, M., FRIEDMAN, M. & KLIMES, J. (2009) Highly variable patterns of antimicrobial resistance in commensal

Escherichia coli isolates from pigs, sympatric rodents, and flies. *Microb Drug Resist,* 15, 229-37.
LIU, H., MAGOUN, L., LUPERCHIO, S., SCHAUER, D. B. & LEONG, J. M. (1999) The Tir-binding region of enterohaemorrhagic *Escherichia coli* intimin is sufficient to trigger actin condensation after bacterial-induced host cell signalling. *Mol Microbiol,* 34, 67-81.
LLOYD, S. A., FORSBERG, A., WOLF-WATZ, H. & FRANCIS, M. S. (2001a) Targeting exported substrates to the *Yersinia* TTSS: different functions for different signals? *Trends Microbiol,* 9, 367-371.
LLOYD, S. A., NORMAN, M., ROSQVIST, R. & WOLF-WATZ, H. (2001b) *Yersinia* YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol Microbiol,* 39, 520-31.
LLOYD, S. A., SJOSTROM, M., ANDERSSON, S. & WOLF-WATZ, H. (2002) Molecular characterization of type III secretion signals via analysis of synthetic N-terminal amino acid sequences. *Mol Microbiol,* 43, 51-59.
LODATO, P. B. & KAPER, J. B. (2009) Post-transcriptional processing of the LEE4 operon in enterohaemorrhagic *Escherichia coli*. *Mol Microbiol,* 71, 273-90.
LOW AS, H. N., ROSSER T, ROE AJ, CONSTANTINIDOU C, HOBMAN JL, SMITH DG, LOW JC, GALLY DL (2006) Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic *Escherichia coli* O157:H7. *Environ Microbiol,* 8, 1033-47.
LUCCHETTI-MIGANEH, C., BURROWES, E., BAYSSE, C. & ERMEL, G. (2008) The post-transcriptional regulator CsrA plays a central role in the adaptation of bacterial pathogens to different stages of infection in animal hosts. *Microbiology,* 154, 16-29.
LUO, Y., BERTERO, M. G., FREY, E. A., PFUETZNER, R. A., WENK, M. R., CREAGH, L., MARCUS, S. L., LIM, D., SICHERI, F., KAY, C., HAYNES, C., FINLAY, B. B. & STRYNADKA, N. C. (2001) Structural and biochemical characterization of the type III secretion chaperones CesT and SigE. *Nat Struct Biol,* 8, 1031-6.
LUPERCHIO, S. A., NEWMAN, J. V., DANGLER, C. A., SCHRENZEL, M. D., BRENNER, D. J., STEIGERWALT, A. G. & SCHAUER, D. B. (2000) *Citrobacter rodentium*, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: synonymy of C. rodentium and mouse-pathogenic *Escherichia coli*. *J Clin Microbiol,* 38, 4343-50.
LUPERCHIO, S. A. & SCHAUER, D. B. (2001) Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. *Microbes Infect,* 3, 333-40.
MAIER, B., CHEN, I., DUBNAU, D. & SHEETZ, M. P. (2004) DNA transport into *Bacillus subtilis* requires proton motive force to generate large molecular forces. *Nat Struct Mol Biol,* 11, 643-9.
MAJDALANI, N., VANDERPOOL, C. K. & GOTTESMAN, S. (2005) Bacterial small RNA regulators. *Crit Rev Biochem Mol Biol,* 40, 93-113.
MAKISHIMA, S., KOMORIYA, K., YAMAGUCHI, S. & AIZAWA, S. I. (2001) Length of the flagellar hook and the capacity of the type III export apparatus. *Science,* 291, 2411-3.
MARCHES, O., LEDGER, T. N., BOURY, M., OHARA, M., TU, X., GOFFAUX, F., MAINIL, J., ROSENSHINE, I., SUGAI, M., DE RYCKE, J. &

OSWALD, E. (2003) Enteropathogenic and enterohaemorrhagic *Escherichia coli* deliver a novel effector called Cif, which blocks cell cycle G2/M transition. *Mol Microbiol,* 50, 1553-67.
MARLEY, M. G., MEGANATHAN, R. & BENTLEY, R. (1986) Menaquinone (vitamin K2) biosynthesis in *Escherichia coli*: synthesis of o-succinylbenzoate does not require the decarboxylase activity of the ketoglutarate dehydrogenase complex. *Biochemistry,* 25, 1304-7.
MARLOVITS, T. C., KUBORI, T., SUKHAN, A., THOMAS, D. R., GALAN, J. E. & UNGER, V. M. (2004) Structural insights into the assembly of the type III secretion needle complex. *Science,* 306, 1040-2.
MATSUZAWA, T., KUWAE, A. & ABE, A. (2005) Enteropathogenic *Escherichia coli* type III effectors EspG and EspG2 alter epithelial paracellular permeability. *Infect Immun,* 73, 6283-9.
MATZURA, O. & WENNBORG, A. (1996) RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Comput Appl Biosci,* 12, 247-9.
MAURELLI, A. T., FERNANDEZ, R. E., BLOCH, C. A., RODE, C. K. & FASANO, A. (1998) "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Proc Natl Acad Sci U S A,* 95, 3943-8.
MCCARTHY, M. (1996) *E. coli* O157:H7 outbreak in USA traced to apple juice. *Lancet,* 348, 1299.
MCCAW, M. L., LYKKEN, G. L., SINGH, P. K. & YAHR, T. L. (2002) ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol Microbiol,* 46, 1123-33.
MCDANIEL, T. K., JARVIS, K. G., DONNENBERG, M. S. & KAPER, J. B. (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci U S A,* 92, 1664-8.
MCDANIEL, T. K. & KAPER, J. B. (1997) A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *Escherichia coli* K-12. *Mol Microbiol,* 23, 399-407.
MCINTYRE, L., FUNG, J., PACCAGNELLA, A., ISAAC-RENTON, J., ROCKWELL, F., EMERSON, B. & PRESTON, T. (2002) *Escherichia coli* O157 outbreak associated with the ingestion of unpasteurized goat's milk in British Columbia, 2001. *Can Commun Dis Rep,* 28, 6-8.
MCNALLY, A., ROE, A. J., SIMPSON, S., THOMSON-CARTER, F. M., HOEY, D. E. E., CURRIE, C., CHAKRABORTY, T., SMITH, D. G. E. & GALLY, D. L. (2001) Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. *Infect Immun,* 69, 5107-5114.
MCNAMARA, B. P. & DONNENBERG, M. S. (1998) A novel proline-rich protein, EspF, is secreted from enteropathogenic *Escherichia coli* via the type III export pathway. *FEMS Microbiol Lett,* 166, 71-78.
MECSAS, J. J. & STRAUSS, E. J. (1996) Molecular mechanisms of bacterial virulence: type III secretion and pathogenicity islands. *Emerg Infect Dis,* 2, 270-88.
MELLIES, J. L., BARRON, A. M. & CARMONA, A. M. (2007) Enteropathogenic and enterohemorrhagic *Escherichia coli* virulence gene regulation. *Infect Immun,* 75, 4199-210.

MELLIES, J. L., ELLIOTT, S. J., SPERANDIO, V., DONNENBERG, M. S. & KAPER, J. B. (1999) The Per regulon of enteropathogenic *Escherichia coli* : identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol Microbiol,* 33, 296-306.
MENARD, R., SANSONETTI, P. & PARSOT, C. (1994) The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J,* 13, 5293-302.
MIAO, E. A., MILLER, S.I. (2000) A conserved amino acid sequence directing intracellular type III secretion by *Escherichia coli*. *Proc Natl Acad Sci U S A,* 97, 7539-7544.
MICHIELS, T. & CORNELIS, G. R. (1991) Secretion of hybrid proteins by the *Yersinia* Yop export system. *J Bacteriol,* 173, 1677-1685.
MICHIELS, T., WATTIAU, P., BRASSEUR, R., RUYSSCHAERT, J. M. & CORNELIS, G. (1990) Secretion of Yop Proteins By *Yersinia*. *Infect Immun,* 58, 2840-2849.
MICHINO, H., ARAKI, K., MINAMI, S., TAKAYA, S., SAKAI, N., MIYAZAKI, M., ONO, A. & YANAGAWA, H. (1999) Massive outbreak of *Escherichia coli* O157:H7 infection in school children in Sakai City, Japan, associated with consumption of white radish sprouts. *Am J Epidemiol,* 150, 787-796.
MIKI, T., SHIBAGAKI, Y., DANBARA, H. & OKADA, N. (2009) Functional characterization of SsaE, a novel chaperone protein of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *J Bacteriol,* 191, 6843-54.
MILLS, E., BARUCH, K., CHARPENTIER, X., KOBI, S. & ROSENSHINE, I. (2008) Real-time analysis of effector translocation by the type III secretion system of enteropathogenic *Escherichia coli*. *Cell Host Microbe,* 3, 104-13.
MILNE, L. M., PLOM, A., STRUDLEY, I., PRITCHARD, G. C., CROOKS, R., HALL, M., DUCKWORTH, G., SENG, C., SUSMAN, M. D., KEARNEY, J., WIGGINS, R. J., MOULSDALE, M., CHEASTY, T. & WILLSHAW, G. A. (1999) *Escherichia coli* O157 incident associated with a farm open to members of the public. *Commun Dis Public Health,* 2, 22-6.
MINAMINO, T. & MACNAB, R. M. (2000) Domain structure of *Salmonella* FlhB, a flagellar export component responsible for substrate specificity switching. *J Bacteriol,* 182, 4906-14.
MINAMINO, T. & NAMBA, K. (2008) Distinct roles of the FliI ATPase and proton motive force in bacterial flagellar protein export. *Nature,* 451, 485-8.
MINAMINO, T., SAIJO-HAMANO, Y., FURUKAWA, Y., GONZALEZ-PEDRAJO, B., MACNAB, R. M. & NAMBA, K. (2004) Domain organization and function of *Salmonella* FliK, a flagellar hook-length control protein. *J Mol Biol,* 341, 491-502.
MIYAZAKI, J., BA-THEIN, W., KUMAO, T., OBATA YASUOKA, M., AKAZA, H. & HAYSHI, H. (2002) Type 1, P and S fimbriae, and a fimbrial adhesin I are not essential for uropathogenic *Escherichia coli* to adhere to and invade bladder epithelial cells. *FEMS Immunol Med Microbiol,* 33, 23-6.
MOHANTY, B. K., MAPLES, V. F. & KUSHNER, S. R. (2004) The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol,* 54, 905-20.

MOLL, I., AFONYUSHKIN, T., VYTVYTSKA, O., KABERDIN, V. R. & BLASI, U. (2003a) Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA,* 9, 1308-1314.
MOLL, I., LEITSCH, D., STEINHAUSER, T. & BLASI, U. (2003b) RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep,* 4, 284-9.
MOON, H. W., WHIPP, S. C., ARGENZIO, R. A., LEVINE, M. M. & GIANNELLA, R. A. (1983) Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect Immun,* 41, 1340-51.
MORABITO, S., TOZZOLI, R., OSWALD, E. & CAPRIOLI, A. (2003) A mosaic pathogenicity island made up of the locus of enterocyte effacement and a pathogenicity island of *Escherichia coli* O157:H7 is frequently present in attaching and effacing *Escherichia coli*. *Infect Immun,* 71, 3343-8.
MORIEL, D. G., BERTOLDI, I., SPAGNUOLO, A., MARCHI, S., ROSINI, R., NESTA, B., PASTORELLO, I., COREA, V. A., TORRICELLI, G., CARTOCCI, E., SAVINO, S., SCARSELLI, M., DOBRINDT, U., HACKER, J., TETTELIN, H., TALLON, L. J., SULLIVAN, S., WIELER, L. H., EWERS, C., PICKARD, D., DOUGAN, G., FONTANA, M. R., RAPPUOLI, R., PIZZA, M. & SERINO, L. (2010) Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A,* 107, 9072-7.
MORIYA, N., MINAMINO, T., HUGHES, K. T., MACNAB, R. M. & NAMBA, K. (2006) The type III flagellar export specificity switch is dependent on FliK ruler and a molecular clock. *J Mol Biol,* 359, 466-77.
MOSS, M. (2009) *Escherichia coli* Outbreak Traced to Company That Halted Testing of Ground Beef Trimmings *The New York Times.* New York.
MUELLER, C. A., BROZ, P., MULLER, S. A., RINGLER, P., ERNE-BRAND, F., SORG, I., KUHN, M., ENGEL, A. & CORNELIS, G. R. (2005) The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles. *Science,* 310, 674-6.
MUFFLER, A., FISCHER, D. & HENGGE-ARONIS, R. (1996) The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for rpoS translation in *Escherichia coli*. *Genes Dev,* 10, 1143-51.
MULVEY, M. A., SCHILLING, J. D., MARTINEZ, J. J. & HULTGREN, S. J. (2000) Bad bugs and beleaguered bladders: interplay between uropathogenic *Escherichia coli* and innate host defenses. *Proc Natl Acad Sci U S A,* 97, 8829-35.
MUNDY, R., JENKINS, C., YU, J., SMITH, H. & FRANKEL, G. (2004a) Distribution of *espI* among clinical enterohaemorrhagic and enteropathogenic *Escherichia coli* isolates. *J Med Microbiol,* 53, 1145-9.
MUNDY, R., PETROVSKA, L., SMOLLETT, K., SIMPSON, N., WILSON, R. K., YU, J., TU, X., ROSENSHINE, I., CLARE, S., DOUGAN, G. & FRANKEL, G. (2004b) Identification of a novel *Citrobacter rodentium* type III secreted protein, EspI, and roles of this and other secreted proteins in infection. *Infect Immun,* 72, 2288-302.
MUTO, T., NAKAGAWA, M., ISOBE, Y., SAITO, M. & NAKANO, T. (1969) Infectious megaenteron of mice. I. Manifestation and pathological observation. *Jpn J Med Sci Biol,* 22, 363-74.

NADLER, C., SHIFRIN, Y., NOV, S., KOBI, S. & ROSENSHINE, I. (2006) Characterization of enteropathogenic *Escherichia coli* mutants that fail to disrupt host cell spreading and attachment to substratum. *Infect Immun,* 74, 839-49.
NAGY, B. & FEKETE, P. Z. (1999) Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. *Vet Res,* 30, 259-284.
NATARO, J. P. & KAPER, J. B. (1998) Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev,* 11, 143-201.
NAVANEETHAN, U. & GIANNELLA, R. A. (2008) Mechanisms of infectious diarrhea. *Nat Clin Pract Gastroenterol Hepatol,* 5, 637-47.
NAYLOR, S. W., LOW, J. C., BESSER, T. E., MAHAJAN, A., GUNN, G. J., PEARCE, M. C., MCKENDRICK, I. J., SMITH, D. G. & GALLY, D. L. (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun,* 71, 1505-12.
NAYLOR, S. W., ROE, A. J., NART, P., SPEARS, K., SMITH, D. G., LOW, J. C. & GALLY, D. L. (2005) *Escherichia coli* O157 : H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology,* 151, 2773-81.
NEVES, B. C., MUNDY, R., PETROVSKA, L., DOUGAN, G., KNUTTON, S. & FRANKEL, G. (2003a) CesD2 of enteropathogenic *Escherichia coli* is a second chaperone for the type III secretion translocator protein EspD. *Infect Immun,* 71, 2130-41.
NEVES, B. C., SHAW, R. K., FRANKEL, G. & KNUTTON, S. (2003b) Polymorphisms within EspA filaments of enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun,* 71, 2262-5.
NEYT, C. & CORNELIS, G. R. (1999) Role of SycD, the chaperone of the *Yersinia* Yop translocators YopB and YopD. *Mol Microbiol,* 31, 143-56.
NGUYEN, L., PAULSEN, I. T., TCHIEU, J., HUECK, C. J. & SAIER, M. H., JR. (2000) Phylogenetic analyses of the constituents of Type III protein secretion systems. *J Mol Microbiol Biotechnol,* 2, 125-44.
NISAN, I., WOLFF, C., HANSKI, E. & ROSENSHINE, I. (1998) Interaction of enteropathogenic *Escherichia coli* with host epithelial cells. *Folia Microbiol (Praha),* 43, 247-52.
NISSINEN, R. M., YTTERBERG, A. J., BOGDANOVE, A. J., KJ, V. A. N. W. & BEER, S. V. (2007) Analyses of the secretomes of *Erwinia amylovora* and selected hrp mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. *Mol Plant Pathol,* 8, 55-67.
NOEL, J. M. & BOEDEKER, E. C. (1997) Enterohemorrhagic *Escherichia coli*: A family of emerging pathogens. *Dig Dis,* 15, 67-91.
NOGUEIRA, T. & SPRINGER, M. (2000) Post-transcriptional control by global regulators of gene expression in bacteria. *Curr Opin Microbiol,* 3, 154-8.
NUCCIO, S. P. & BAUMLER, A. J. (2007) Evolution of the chaperone/usher assembly pathway: fimbrial classification goes Greek. *Microbiol Mol Biol Rev,* 71, 551-75.
O'BRIEN, A. D. & LAVECK, G. D. (1983) Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. *Infect Immun,* 40, 675-83.

O'BRIEN, A. D., LAVECK, G. D., THOMPSON, M. R. & FORMAL, S. B. (1982) Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. *J Infect Dis,* 146, 763-9.
O'BRIEN, A. D., LIVELY, T. A., CHANG, T. W. & GORBACH, S. L. (1983) Purification of *Shigella dysenteriae* 1 (Shiga)-like toxin from *Escherichia coli* O157:H7 strain associated with haemorrhagic colitis. *Lancet,* 2, 573.
O'BRIEN, A. D., NEWLAND, J. W., MILLER, S. F., HOLMES, R. K., SMITH, H. W. & FORMAL, S. B. (1984) Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science,* 226, 694-696.
O'BRIEN, S. J. (2005) Foodborne zoonoses. *BMJ,* 331, 1217-8.
O'CONNELL, C. B., CREASEY, E. A., KNUTTON, S., ELLIOTT, S., CROWTHER, L. J., LUO, W., ALBERT, M. J., KAPER, J. B., FRANKEL, G. & DONNENBERG, M. S. (2004) SepL, a protein required for enteropathogenic *Escherichia coli* type III translocation, interacts with secretion component SepD. *Mol Microbiol,* 52, 1613-25.
OGAWA, M., HANDA, Y., ASHIDA, H., SUZUKI, M. & SASAKAWA, C. (2008) The versatility of *Shigella* effectors. *Nat Rev Microbiol,* 6, 11-6.
OGINO, T., OHNO, R., SEKIYA, K., KUWAE, A., MATSUZAWA, T., NONAKA, T., FUKUDA, H., IMAJOH-OHMI, S. & ABE, A. (2006) Assembly of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *J Bacteriol,* 188, 2801-11.
OHSUMI, Y. & ANRAKU, Y. (1983) Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J Biol Chem,* 258, 5614-7.
OKOH, A. I. & OSODE, A. N. (2008) Enterotoxigenic *Escherichia coli* (ETEC): a recurring decimal in infants' and travelers' diarrhea. *Rev Environ Health,* 23, 135-48.
OKREND, A. J. G., ROSE, B. E. & BENNETT, B. (1990) A screening method for the isolation of *Escherichia coli* O157:H7 from ground-beef. *J Food Prot,* 53, 249-252.
OLGAARD, K., MADSEN, S. & JACOBSEN, S. V. (1974) Recurrent attacks of hemolytic anemia, thrombocytopenia and uremia--hemolytic uremic syndrome? *Acta Med Scand,* 196, 541-3.
ONG, C. L., ULETT, G. C., MABBETT, A. N., BEATSON, S. A., WEBB, R. I., MONAGHAN, W., NIMMO, G. R., LOOKE, D. F., MCEWAN, A. G. & SCHEMBRI, M. A. (2008) Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. *J Bacteriol,* 190, 1054-63.
ORRETT, F. A. & CHANGOOR, E. (2007) Bacteremia in children at a regional hospital in Trinidad. *Int J Infect Dis,* 11, 145-51.
ORSKOV, F. & ORSKOV, I. (1990) The serology of capsular antigens. *Curr Top Microbiol Immunol,* 150, 43-63.
ORSKOV, F. & ORSKOV, I. (1992) *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol,* 38, 699-704.
ORSKOV, F., ORSKOV, I. & VILLAR, J. A. (1987) Cattle as a reservoir of verotoxin-producing *Escherichia coli* O157:H7. *Lancet,* 1, 276.
ORSKOV, I. & ORSKOV, F. (1985) *Escherichia coli* in extra-intestinal infections. *J Hyg (Lond),* 95, 551-75.

OSTROFF, S. M., GRIFFIN, P. M., TAUXE, R. V., SHIPMAN, L. D., GREENE, K. D., WELLS, J. G., LEWIS, J. H., BLAKE, P. A. & KOBAYASHI, J. M. (1990) A statewide outbreak of *Escherichia coli* O157:H7 infections in Washington State. *Am J Epidemiol,* 132, 239-47.
PADHYE, N. V. & DOYLE, M. P. (1991) Rapid procedure for detecting enterohemorrhagic *Escherichia coli* O157-H7 in food. *Appl Environ Microbiol,* 57, 2693-2698.
PALLECCHI, L., RICCOBONO, E., SENNATI, S., MANTELLA, A., BARTALESI, F., TRIGOSO, C., GOTUZZO, E., BARTOLONI, A. & ROSSOLINI, G. M. (2010) Characterization of small ColE-like plasmids mediating widespread dissemination of the qnrB19 gene in commensal enterobacteria. *Antimicrob Agents Chemother,* 54, 678-82.
PALLEN, M., CHAUDHURI, R. & KHAN, A. (2002) Bacterial FHA domains: neglected players in the phospho-threonine signalling game? *Trends Microbiol,* 10, 556-63.
PALLEN, M. J., BEATSON, S. A. & BAILEY, C. M. (2005a) Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion. *BMC Microbiol,* 5, 9.
PALLEN, M. J., BEATSON, S. A. & BAILEY, C. M. (2005b) Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perspective. *FEMS Microbiol Rev,* 29, 201-29.
PALLEN, M. J., FRANCIS, M. S. & FUTTERER, K. (2003) Tetratricopeptide-like repeats in type-III-secretion chaperones and regulators. *FEMS Microbiol Lett,* 223, 53-60.
PAPENFORT, K., SAID, N., WELSINK, T., LUCCHINI, S., HINTON, J. C. & VOGEL, J. (2009) Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. *Mol Microbiol,* 74, 139-158.
PARSOT, C. (2005) *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. *FEMS Microbiol Lett,* 252, 11-8.
PARSOT, C., HAMIAUX, C. & PAGE, A. L. (2003) The various and varying roles of specific chaperones in type III secretion systems. *Curr Opin Microbiol,* 6, 7-14.
PATON, A. W., MANNING, P. A., WOODROW, M. C. & PATON, J. C. (1998) Translocated intimin receptors (Tir) of Shiga-toxigenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from patients with hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. *Infect Immun,* 66, 5580-6.
PAUL, K., ERHARDT, M., HIRANO, T., BLAIR, D. F. & HUGHES, K. T. (2008) Energy source of flagellar type III secretion. *Nature,* 451, 489-92.
PAUL, K., NIETO, V., CARLQUIST, W. C., BLAIR, D. F. & HARSHEY, R. M. (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. *Mol Cell,* 38, 128-39.
PERNA, N. T., MAYHEW, G. F., POSFAI, G., ELLIOTT, S., DONNENBERG, M. S., KAPER, J. B. & BLATTNER, F. R. (1998) Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun,* 66, 3810-3817.
PERNA, N. T., PLUNKETT, G., 3RD, BURLAND, V., MAU, B., GLASNER, J. D., ROSE, D. J., MAYHEW, G. F., EVANS, P. S., GREGOR, J.,

KIRKPATRICK, H. A., POSFAI, G., HACKETT, J., KLINK, S., BOUTIN, A., SHAO, Y., MILLER, L., GROTBECK, E. J., DAVIS, N. W., LIM, A., DIMALANTA, E. T., POTAMOUSIS, K. D., APODACA, J., ANANTHARAMAN, T. S., LIN, J., YEN, G., SCHWARTZ, D. C., WELCH, R. A. & BLATTNER, F. R. (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature,* 409, 529-33.

PHILLIPS, A. D. & FRANKEL, G. (1997) Mechanisms of gut damage by *Escherichia coli*. *Baillieres Clin Gastroenterol,* 11, 465-483.

POLLOCK, K. G., LOCKING, M. E., BEATTIE, T. J., MAXWELL, H., RAMAGE, I., HUGHES, D., COWIESON, J., ALLISON, L., HANSON, M. & COWDEN, J. M. (2010) Sorbitol-fermenting *Escherichia coli* O157, Scotland. *Emerg Infect Dis,* 16, 881-2.

PORTER, M. E., MITCHELL, P., FREE, A., SMITH, D. G. E. & GALLY, D. L. (2005) The LEE1 promoters from both enteropathogenic and enterohemorrhagic *Escherichia coli* can be activated by PerC-like proteins from either organism. *J Bacteriol,* 187, 458-472.

PROCTOR, M. E., KURZYNSKI, T., KOSCHMANN, C., ARCHER, J. R. & DAVIS, J. P. (2002) Four strains of *Escherichia coli* O157:H7 isolated from patients during an outbreak of disease associated with ground beef: importance of evaluating multiple colonies from an outbreak-associated product. *J Clin Microbiol,* 40, 1530-3.

RAJPURA, A., LAMDEN, K., FORSTER, S., CLARKE, S., CHEESBROUGH, J., GORNALL, S. & WATERWORTH, S. (2003) Large outbreak of infection with *Escherichia coli* O157 PT21/28 in Eccleston, Lancashire, due to cross contamination at a butcher's counter. *Commun Dis Public Health,* 6, 279-84.

RAMAMURTHI, K. S. & SCHNEEWIND, O. (2002) *Yersinia enterocolitica* type III secretion: mutational analysis of the yopQ secretion signal. *J Bacteriol,* 184, 3321-8.

RAMAMURTHI, K. S. & SCHNEEWIND, O. (2003a) Substrate recognition by the *Yersinia* type III protein secretion machinery. *Mol Microbiol,* 50, 1095-102.

RAMAMURTHI, K. S. & SCHNEEWIND, O. (2003b) *Yersinia* yopQ mRNA encodes a bipartite type III secretion signal in the first 15 codons. *Mol Microbiol,* 50, 1189-98.

RANGEL, J. M., SPARLING, P. H., CROWE, C., GRIFFIN, P. M. & SWERDLOW, D. L. (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg Infect Dis,* 11, 603-609.

REIGSTAD, C. S., HULTGREN, S. J. & GORDON, J. I. (2007) Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. *J Biol Chem,* 282, 21259-67.

RICHARDSON, S. E., KARMALI, M. A., BECKER, L. E. & SMITH, C. R. (1988) The Hisopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. *Hum Pathol,* 19, 1102-1108.

RILEY, L. W., REMIS, R. S., HELGERSON, S. D., MCGEE, H. B., WELLS, J. G., DAVIS, B. R., HEBERT, R. J., OLCOTT, E. S., JOHNSON, L. M., HARGRETT, N. T., BLAKE, P. A. & COHEN, M. L. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med,* 308, 681-685.

RIORDAN, K. E. & SCHNEEWIND, O. (2008) YscU cleavage and the assembly of *Yersinia* type III secretion machine complexes. *Mol Microbiol,* 68, 1485-501.
RIVAS, M., VOYER, L. E., TOUS, M., DEMENA, M. F., LEARDINI, N., WAINSZTEIN, R., CALLEJO, R., QUADRI, B., CORTI, S. & PRADO, V. (1996) Verocytotoxin-producing *Escherichia coli* infection in family members of children with hemolytic uremic syndrome. *Medicina-Buenos Aires,* 56, 119-125.
RODGERS, L., GAMEZ, A., RIEK, R. & GHOSH, P. (2008) The type III secretion chaperone SycE promotes a localized disorder-to-order transition in the natively unfolded effector YopE. *J Biol Chem,* 283, 20857-63.
RODRIGUEZ-PASTRANA, A. F., LOPEZ-SAUCEDO, C., SALAZAR-EXAIRE, D. & ESTRADA-GARCIA, T. (2007) Patient's UPEC isolates fim+/pap- only correlated with low UTI whereas pyelonephritis isolates were fim+/pap+. *Kidney Int,* 72, 1289.
ROE, A. J., GALLY, D.L. (2000) Enteropathogenic and enterohaemorrhagic *Escherichia coli* and diarrhoea. *Curr Opin Infect Dis,* 13, 511 - 517.
ROE, A. J., HOEY, D. E. & GALLY, D. L. (2003a) Regulation, secretion and activity of type III-secreted proteins of enterohaemorrhagic *Escherichia coli* O157. *Biochem Soc Trans,* 31, 98-103.
ROE, A. J., NAYLOR, S. W., SPEARS, K. J., YULL, H. M., DRANSFIELD, T. A., OXFORD, M., MCKENDRICK, I. J., PORTER, M., WOODWARD, M. J., SMITH, D. G. & GALLY, D. L. (2004) Co-ordinate single-cell expression of LEE4- and LEE5-encoded proteins of *Escherichia coli* O157:H7. *Mol Microbiol,* 54, 337-52.
ROE, A. J., TYSALL, L., DRANSFIELD, T., WANG, D., FRASER-PITT, D., MAHAJAN, A., CONSTANDINOU, C., INGLIS, N., DOWNING, A., TALBOT, R., SMITH, D. G. & GALLY, D. L. (2007) Analysis of the expression, regulation and export of NleA-E in *Escherichia coli* O157 : H7. *Microbiology,* 153, 1350-60.
ROE, A. J., YULL, H., NAYLOR, S. W., WOODWARD, M. J., SMITH, D. G. E. & GALLY, D. L. (2003b) Heterogeneous surface expression of EspA translocon filaments by *Escherichia coli* O157:H7 is controlled at the posttranscriptional level. *Infect Immun,* 71, 5900-5909.
ROMEO, T. (1996) Post-transcriptional regulation of bacterial carbohydrate metabolism: evidence that the gene product CsrA is a global mRNA decay factor. *Res Microbiol,* 147, 505-12.
ROMEO, T. (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol,* 29, 1321-30.
ROSA, A. C. P., MARIANO, A. T., PEREIRA, A. M. S., TIBANA, A., GOMES, T. A. T. & ANDRADE, J. R. C. (1998) Enteropathogenicity markers in *Escherichia coli* isolated from infants with acute diarrhoea and healthy controls in Rio de Janeiro, Brazil. *J Med Microbiol,* 47, 781-790.
ROSENSHINE, I., DONNENBERG, M. S., KAPER, J. B. & FINLAY, B. B. (1992) Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO J,* 11, 3551-60.

ROSENSHINE, I., RUSCHKOWSKI, S. & FINLAY, B. B. (1996a) Expression of attaching effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. *Infect Immun,* 64, 966-973.
ROSENSHINE, I., RUSCHKOWSKI, S., STEIN, M., REINSCHEID, D. J., MILLS, S. D. & FINLAY, B. B. (1996b) A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J,* 15, 2613-2624.
ROSENZWEIG, J. A., CHROMY, B., ECHEVERRY, A., YANG, J., ADKINS, B., PLANO, G. V., MCCUTCHEN-MALONEY, S. & SCHESSER, K. (2007) Polynucleotide phosphorylase independently controls virulence factor expression levels and export in *Yersinia* spp. *FEMS Microbiol Lett,* 270, 255-64.
ROSENZWEIG, J. A. & SCHESSER, K. (2007) Polynucleotide phosphorylase and the T3SS. *Adv Exp Med Biol,* 603, 217-24.
ROSQVIST, R., MAGNUSSON, K. E. & WOLF-WATZ, H. (1994) Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J,* 13, 964-72.
ROSQVIST, R., PERSSON, C., HAKANSSON, S., NORDFELDT, R. & WOLF-WATZ, H. (1995) Translocation of the *Yersinia* YopE and YopH virulence proteins into target cells is mediated by YopB and YopD. *Contrib Microbiol Immunol,* 13, 230-4.
RUSSO, E. (2003) The birth of biotechnology. *Nature,* 421, 456-7.
RUSSO, T. A. & JOHNSON, J. R. (2006) Extraintestinal isolates of *Escherichia coli*: identification and prospects for vaccine development. *Expert Rev Vaccines,* 5, 45-54.
RYAN, C. A., TAUXE, R. V., HOSEK, G. W., WELLS, J. G., STOESZ, P. A., MCFADDEN, H. W., JR., SMITH, P. W., WRIGHT, G. F. & BLAKE, P. A. (1986) *Escherichia coli* O157:H7 diarrhea in a nursing home: clinical, epidemiological, and pathological findings. *J Infect Dis,* 154, 631-8.
RYAN KJ, R. C. (Ed.) (2004) *Sherris Medical Microbiolgy* McGraw Hill.
SAIER, M. H., JR. (2004) Evolution of bacterial type III protein secretion systems. *Trends Microbiol,* 12, 113-5.
SALMOND, G. P. & REEVES, P. J. (1993) Membrane traffic wardens and protein secretion in gram-negative bacteria. *Trends Biochem Sci,* 18, 7-12.
SALYERS, A. A., GUPTA, A. & WANG, Y. (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol,* 12, 412-6.
SAMBROOK, S., FRITSCH, E. F. & MANIATIS, T. (1989) *Molecular Cloning: A Laboratory Manual,* New York, Cold Spring Harbour Laboratory Press.
SANCHEZ-SANMARTIN, C., BUSTAMANTE, V. W., CALVA, E. & PUENTE, J. L. (2001) Transcriptional regulation of the *orf19* gene and the *tir-cesT- eae* operon of enteropathogenic *Escherichia coli*. *J Bacteriol,* 183, 2823-2833.
SANSONETTI, P. (1999) *Shigella* plays dangerous games. *ASM News,* 65, 611-617.
SARKAR, M. K., PAUL, K. & BLAIR, D. (2010) Chemotaxis signaling protein CheY binds to the rotor protein FliN to control the direction of flagellar rotation in *Escherichia coli*. *Proc Natl Acad Sci U S A,* 107, 9370-5.

SAUTER, C., BASQUIN, J. & SUCK, D. (2003) Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from *Escherichia coli*. *Nucleic Acids Res,* 31, 4091-8.
SCHAUDER, S., SHOKAT, K., SURETTE, M. G. & BASSLER, B. L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol,* 41, 463-476.
SCHAUER, D. B., ZABEL, B. A., PEDRAZA, I. F., O'HARA, C. M., STEIGERWALT, A. G. & BRENNER, D. J. (1995) Genetic and biochemical characterization of *Citrobacter rodentium* sp. nov. *J Clin Microbiol,* 33, 2064-8.
SCHESSER, K., FRITHZ-KINDSTEN, E. & WOLF-WATZ, H. (1996) Delineation ans mutational analysis of the *Yersinia pseudotuberculosis* YopE domians which mediate translocation across bacterial and eukaryotic cellular membranes. *J Bacteriol,* 178, 7227-7233.
SCHROEDER, G. N. & HILBI, H. (2008) Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev,* 21, 134-56.
SCHUBOT, F. D., JACKSON, M. W., PENROSE, K. J., CHERRY, S., TROPEA, J. E., PLANO, G. V. & WAUGH, D. S. (2005) Three-dimensional structure of a macromolecular assembly that regulates type III secretion in *Yersinia pestis*. *J Mol Biol,* 346, 1147-61.
SCHULLER, S., CHONG, Y., LEWIN, J., KENNY, B., FRANKEL, G. & PHILLIPS, A. D. (2007) Tir phosphorylation and Nck/N-WASP recruitment by enteropathogenic and enterohaemorrhagic *Escherichia coli* during ex vivo colonization of human intestinal mucosa is different to cell culture models. *Cell Microbiol,* 9, 1352-64.
SCHULLER, S., FRANKEL, G. & PHILLIPS, A. D. (2004) Interaction of Shiga toxin from *Escherichia coli* with human intestinal epithelial cell lines and explants: Stx2 induces epithelial damage in organ culture. *Cell Microbiol,* 6, 289-301.
SEED, P. C. & HULTGREN, S. J. (2005) Blueprinting the regulatory response of *Escherichia coli* to the urinary tract. *Trends Microbiol,* 13, 246-8.
SEKIYA, K., OHISHI, M., OGINO, T., TAMANO, K., SASAKAWA, C. & ABE, A. (2001) Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proc Natl Acad Sci U S A,* 98, 11638-43.
SERVICES, O. D. O. H. (2003) Hemorrhagic Escherichiosis from a County Fair. *CD Summary,* 51.
SHAKHNOVICH, E. A., DAVIS, B. M. & WALDOR, M. K. (2009) Hfq negatively regulates type III secretion in EHEC and several other pathogens. *Mol Microbiol,* 74, 347-63.
SHAPIRO, L., FRANZE DE FERNANDEZ, M. T. & AUGUST, J. T. (1968) Resolution of two factors required in the Q-beta-RNA polymerase reaction. *Nature,* 220, 478-80.
SHARMA, V. K. & ZUERNER, R. L. (2004) Role of *hha* and *ler* in transcriptional regulation of the *esp* operon of enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol,* 186, 7290-301.
SHARP, F. C. & SPERANDIO, V. (2007) QseA directly activates transcription of LEE1 in enterohemorrhagic *Escherichia coli*. *Infect Immun,* 75, 2432-40.

SHAW, R. K., DANIELL, S., EBEL, F., FRANKEL, G. & KNUTTON, S. (2001) EspA filament-mediated protein translocation into red blood cells. *Cell Microbiol,* 3, 213-222.
SHAW, R. K., DANIELL, S., FRANKEL, G. & KNUTTON, S. (2002) Enteropathogenic *Escherichia coli* translocate Tir and form an intimin-Tir intimate attachment to red blood cell membranes. *Microbiology,* 148, 1355-1365.
SHELDON, I. M., RYCROFT, A. N., DOGAN, B., CRAVEN, M., BROMFIELD, J. J., CHANDLER, A., ROBERTS, M. H., PRICE, S. B., GILBERT, R. O. & SIMPSON, K. W. (2010) Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS One,* 5, e9192.
SHIBATA, S., TAKAHASHI, N., CHEVANCE, F. F., KARLINSEY, J. E., HUGHES, K. T. & AIZAWA, S. (2007) FliK regulates flagellar hook length as an internal ruler. *Mol Microbiol,* 64, 1404-15.
SHIGA, K. (1898) Ueber den Erreger der Dysenterie in Japan. *Zentralbl Bakteriol Mikrobiol Hyg (Vorläufige Mitteilung)* 23, 599-600.
SHPIGEL, N. Y., ELAZAR, S. & ROSENSHINE, I. (2008) Mammary pathogenic *Escherichia coli*. *Curr Opin Microbiol,* 11, 60-5.
SHUKLA, R., SLACK, R., GEORGE, A., CHEASTY, T., ROWE, B. & SCUTTER, J. (1995) *Escherichia coli* O157 infection associated with a farm visitor centre. *Commun Dis Rep CDR Rev,* 5, R86-90.
SIMEONE, R., BOTTAI, D. & BROSCH, R. (2009) ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol,* 12, 4-10.
SIMMS, A. N. & MOBLEY, H. L. (2008a) Multiple genes repress motility in uropathogenic *Escherichia coli* constitutively expressing type 1 fimbriae. *J Bacteriol,* 190, 3747-56.
SIMMS, A. N. & MOBLEY, H. L. (2008b) PapX, a P fimbrial operon-encoded inhibitor of motility in uropathogenic *Escherichia coli*. *Infect Immun,* 76, 4833-41.
SINCLAIR, J. F., DEAN-NYSTROM, E. A. & O'BRIEN, A. D. (2006) The established intimin receptor Tir and the putative eucaryotic intimin receptors nucleolin and beta1 integrin localize at or near the site of enterohemorrhagic *Escherichia coli* O157:H7 adherence to enterocytes *in vivo*. *Infect Immun,* 74, 1255-65.
SINCLAIR, J. F. & O'BRIEN, A. D. (2002) Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157 : H7. *J Biol Chem,* 277, 2876-2885.
SINCLAIR, J. F. & O'BRIEN, A. D. (2004) Intimin types alpha, beta, and gamma bind to nucleolin with equivalent affinity but lower avidity than to the translocated intimin receptor. *J Biol Chem,* 279, 33751-8.
SITTKA, A., LUCCHINI, S., PAPENFORT, K., SHARMA, C. M., ROLLE, K., BINNEWIES, T. T., HINTON, J. C. & VOGEL, J. (2008) Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet,* 4, e1000163.
SITTKA, A., SHARMA, C. M., ROLLE, K. & VOGEL, J. (2009) Deep sequencing of *Salmonella* RNA associated with heterologous Hfq proteins *in vivo* reveals small RNAs as a major target class and identifies RNA processing phenotypes. *RNA Biol,* 6, 266-75.

SIVICK, K. E. & MOBLEY, H. L. (2010) Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. *Infect Immun,* 78, 568-85.
SKALA, M. F. (1994) *Escherichia coli* O157:H7 is an emerging pathogen in Missouri. *Mo Med,* 91, 730-3.
SKURNIK, D., LACHEEB, S., BERNEDE, C., LE MENAC'H, A., ELBAZ, S., MOHLER, J., DENAMUR, E., ANDREMONT, A. & RUIMY, R. (2009) Integrons and antibiotic resistance in phylogenetic group B2 *Escherichia coli*. *Microb Drug Resist,* 15, 173-8.
SLEDJESKI, D. D., WHITMAN, C. & ZHANG, A. (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. *J Bacteriol,* 183, 1997-2005.
SMITH, J. L., FRATAMICO, P. M. & GUNTHER, N. W. (2007) Extraintestinal pathogenic *Escherichia coli*. *Foodborne Pathog Dis,* 4, 134-63.
SMITH, Y. C., RASMUSSEN, S. B., GRANDE, K. K., CONRAN, R. M. & O'BRIEN, A. D. (2008) Hemolysin of uropathogenic *Escherichia coli* evokes extensive shedding of the uroepithelium and hemorrhage in bladder tissue within the first 24 hours after intraurethral inoculation of mice. *Infect Immun,* 76, 2978-90.
SONNLEITNER, E., NAPETSCHNIG, J., AFONYUSHKIN, T., ECKER, K., VECEREK, B., MOLL, I., KABERDIN, V. R. & BLASI, U. (2004) Functional effects of variants of the RNA chaperone Hfq. *Biochem Biophys Res Commun,* 323, 1017-23.
SORENSEN, M., LIPPUNER, C., KAISER, T., MISSLITZ, A., AEBISCHER, T. & BUMANN, D. (2003) Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Lett,* 552, 110-4.
SORG, I., WAGNER, S., AMSTUTZ, M., MULLER, S. A., BROZ, P., LUSSI, Y., ENGEL, A. & CORNELIS, G. R. (2007) YscU recognizes translocators as export substrates of the *Yersinia* injectisome. *EMBO J,* 26, 3015-24.
SORG, J. A., MILLER, N. C. & SCHNEEWIND, O. (2005) Substrate recognition of type III secretion machines--testing the RNA signal hypothesis. *Cell Microbiol,* 7, 1217-25.
SORY, M. P., BOLAND, A., LAMBERMONT, I. & CORNELIS, G. R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proc Natl Acad Sci U S A,* 92, 11998-12002.
SPAETH, K. E., CHEN, Y. S. & VALDIVIA, R. H. (2009) The *Chlamydia* type III secretion system C-ring engages a chaperone-effector protein complex. *PLoS Pathog,* 5, e1000579.
SPERANDIO, V., LI, C. Y. C. & KAPER, J. B. (2002) Quorum-sensing *Escherichia coli* regulator A: A regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohemorrhagic *E.coli*. *Infect Immun,* 70, 3085-3093.
SPERANDIO, V., MELLIES, J. L., DELAHAY, R. M., FRANKEL, G., CRAWFORD, J. A., NGUYEN, W. & KAPER, J. B. (2000) Activation of enteropathogenic *Escherichia coli* (EPEC) LEE2 and LEE3 operons by Ler. *Mol Microbiol,* 38, 781-93.
STATHOPOULOS, C., PROVENCE, D. L. & CURTISS, R. (1999) Characterization of the avian pathogenic *Escherichia coli* hemagglutinin Tsh, a member of the immunoglobulin a protease type family of autotransporters. *Infect Immun,* 67, 772-781.

STEBBINS, C. E. & GALAN, J. E. (2001) Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. *Nature,* 414, 77-81.
STORDEUR, P., CHINA, B., CHARLIER, G., ROELS, S. & MAINIL, J. (2000) Clinical signs, reproduction of attaching/effacing lesions, and enterocyte invasion after oral inoculation of an O118 enterohaemorrhagic *Escherichia coli* in neonatal calves. *Microbes Infect,* 2, 17-24.
STORZ, G., OPDYKE, J. A. & ZHANG, A. (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol,* 7, 140-4.
STROBEL, H. J., RUSSELL, J. B., DRIESSEN, A. J. & KONINGS, W. N. (1989) Transport of amino acids in *Lactobacillus casei* by proton-motive-force-dependent and non-proton-motive-force-dependent mechanisms. *J Bacteriol,* 171, 280-4.
SU, C. & BRANDT, L. J. (1995) *Escherichia coli* O157:H7 infection in humans. *Ann Intern Med,* 123, 698-714.
SU, M. S., KAO, H. C., LIN, C. N. & SYU, W. J. (2008) Gene *l0017* encodes a second chaperone for EspA of enterohaemorrhagic *Escherichia coli* O157 : H7. *Microbiology,* 154, 1094-103.
SUKHODOLETS, M. V. & GARGES, S. (2003) Interaction of *Escherichia coli* RNA polymerase with the ribosomal protein S1 and the Sm-like ATPase Hfq. *Biochemistry,* 42, 8022-34.
SUN, X., ZHULIN, I. & WARTELL, R. M. (2002) Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic Acids Res,* 30, 3662-71.
SUNDBERG, L. & FORSBERG, A. (2003) TyeA of *Yersinia pseudotuberculosis* is involved in regulation of Yop expression and is required for polarized translocation of Yop effectors. *Cell Microbiol,* 5, 187-202.
SUZUKI, K., BABITZKE, P., KUSHNER, S. R. & ROMEO, T. (2006) Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. *Genes Dev,* 20, 2605-17.
SWERDLOW, D. L., WOODRUFF, B. A., BRADY, R. C., GRIFFIN, P. M., TIPPEN, S., DONNELL, H. D., JR., GELDREICH, E., PAYNE, B. J., MEYER, A., JR., WELLS, J. G. & *ET AL.* (1992) A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. *Ann Intern Med,* 117, 812-9.
TAMANO, K., AIZAWA, S., KATAYAMA, E., NONAKA, T., IMAJOH-OHMI, S., KUWAE, A., NAGAI, S. & SASAKAWA, C. (2000) Supramolecular structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. *EMBO J,* 19, 3876-87.
TART, A. H., WOLFGANG, M. C. & WOZNIAK, D. J. (2005) The alternative sigma factor AlgT represses *Pseudomonas aeruginosa* flagellum biosynthesis by inhibiting expression of *fleQ*. *J Bacteriol,* 187, 7955-62.
TE LOO, D. M., MONNENS, L. A., VAN DER VELDEN, T. J., VERMEER, M. A., PREYERS, F., DEMACKER, P. N., VAN DEN HEUVEL, L. P. & VAN HINSBERGH, V. W. (2000) Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood,* 95, 3396-402.

TEED, P. (2010) Feltham Hill Nursery and Infant School shut after two pupils diagnosed with E coli. *Richmond and Twickenham Times.* London.
TESH, V. L. & O'BRIEN, A. D. (1991) The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Mol Microbiol,* 5, 1817-22.
THANABALASURIAR, A., KOUTSOURIS, A., WEFLEN, A., MIMEE, M., HECHT, G. & GRUENHEID, S. (2010) The bacterial virulence factor NleA is required for the disruption of intestinal tight junctions by enteropathogenic *Escherichia coli*. *Cell Microbiol*, 12, 31-41.
THANASSI, D. G., SAULINO, E. T. & HULTGREN, S. J. (1998) The chaperone/usher pathway: a major terminal branch of the general secretory pathway. *Curr Opin Microbiol,* 1, 223-31.
THOMAS, J., STAFFORD, G. P. & HUGHES, C. (2004) Docking of cytosolic chaperone-substrate complexes at the membrane ATPase during flagellar type III protein export. *Proc Natl Acad Sci U S A,* 101, 3945-3950.
THOMAS, N. A., DENG, W., BAKER, N., PUENTE, J. & FINLAY, B. B. (2007) Hierarchical delivery of an essential host colonization factor in enteropathogenic *Escherichia coli*. *J Biol Chem,* 282, 29634-45.
THOMAS, N. A., DENG, W., PUENTE, J. L., FREY, E. A., YIP, C. K., STRYNADKA, N. C. & FINLAY, B. B. (2005) CesT is a multi-effector chaperone and recruitment factor required for the efficient type III secretion of both LEE- and non-LEE-encoded effectors of enteropathogenic *Escherichia coli*. *Mol Microbiol,* 57, 1762-79.
THOMAS, N. A. & FINLAY, B. B. (2003) Establishing order for type III secretion substrates - a hierarchical process. *Trends Microbiol,* 11, 398-403.
TOBE, T., BEATSON, S. A., TANIGUCHI, H., ABE, H., BAILEY, C. M., FIVIAN, A., YOUNIS, R., MATTHEWS, S., MARCHES, O., FRANKEL, G., HAYASHI, T. & PALLEN, M. J. (2006) An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proc Natl Acad Sci U S A,* 103, 14941-6.
TOMSON, F. L., VISWANATHAN, V. K., KANACK, K. J., KANTETI, R. P., STRAUB, K. V., MENET, M., KAPER, J. B. & HECHT, G. (2005) Enteropathogenic *Escherichia coli* EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier. *Mol Microbiol,* 56, 447-64.
TORRUELLAS, J., JACKSON, M. W., PENNOCK, J. W. & PLANO, G. V. (2005) The *Yersinia pestis* type III secretion needle plays a role in the regulation of Yop secretion. *Mol Microbiol,* 57, 1719-33.
TREE, J. J., WOLFSON, E. B., WANG, D., ROE, A. J. & GALLY, D. L. (2009) Controlling injection: regulation of type III secretion in enterohaemorrhagic *Escherichia coli*. *Trends Microbiol,* 17, 361-70.
TSAI, N. P., WU, Y. C., CHEN, J. W., WU, C. F., TZENG, C. M. & SYU, W. J. (2006) Multiple functions of l0036 in the regulation of the pathogenicity island of enterohaemorrhagic *Escherichia coli* O157:H7. *Biochem J,* 393, 591-9.
TU, X., NISAN, I., YONA, C., HANSKI, E. & ROSENSHINE, I. (2003) EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic *Escherichia coli*. *Mol Microbiol,* 47, 595-606.
TUTTLE, J., GOMEZ, T., DOYLE, M. P., WELLS, J. G., ZHAO, T., TAUXE, R. V. & GRIFFIN, P. M. (1999) Lessons from a large outbreak of *Escherichia*

coli O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol Infect,* 122, 185-192.
UCHIYA, K., BARBIERI, M. A., FUNATO, K., SHAH, A. H., STAHL, P. D. & GROISMAN, E. A. (1999) A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J,* 18, 3924-33.
ULETT, G. C., MABBETT, A. N., FUNG, K. C., WEBB, R. I. & SCHEMBRI, M. A. (2007) The role of F9 fimbriae of uropathogenic *Escherichia coli* in biofilm formation. *Microbiology,* 153, 2321-31.
UMANSKI, T., ROSENSHINE, I. & FRIEDBERG, D. (2002) Thermoregulated expression of virulence genes in enteropathogenic *Escherichia coli*. *Microbiology,* 148, 2735-44.
URBAN, J. H. & VOGEL, J. (2007) Translational control and target recognition by *Escherichia coli* small RNAs *in vivo*. *Nucleic Acids Res,* 35, 1018-37.
URBAN, J. H. & VOGEL, J. (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. *PLoS Biol,* 6, e64.
URBANOWSKI, M. L., LYKKEN, G. L. & YAHR, T. L. (2005) A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. *Proc Natl Acad Sci U S A,* 102, 9930-5.
VAKULSKAS, C. A., BRADY, K. M. & YAHR, T. L. (2009) Mechanism of transcriptional activation by *Pseudomonas aeruginosa* ExsA. *J Bacteriol,* 191, 6654-64.
VALENTIN-HANSEN, P., ERIKSEN, M. & UDESEN, C. (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol,* 51, 1525-33.
VAN BENEDEN, C. A., KEENE, W. E., STRANG, R. A., WERKER, D. H., KING, A. S., MAHON, B., HEDBERG, K., BELL, A., KELLY, M. T., BALAN, V. K., MAC KENZIE, W. R. & FLEMING, D. (1999) Multinational outbreak of *Salmonella enterica* serotype Newport infections due to contaminated alfalfa sprouts. *JAMA,* 281, 158-62.
VAN DONKERSGOED, J., GRAHAM, T. & GANNON, V. (1999) The prevalence of verotoxins, *Escherichia coli* O157 : H7, and *Salmonella* in the feces and rumen of cattle at processing. *Can Vet J,* 40, 332-338.
VAN LEEUWEN, C. C., POSTMA, E., VAN DEN BROEK, P. J. & VAN STEVENINCK, J. (1991) Proton-motive force-driven D-galactose transport in plasma membrane vesicles from the yeast Kluyveromyces marxianus. *J Biol Chem,* 266, 12146-51.
VEESENMEYER, J. L., HOWELL, H., HALAVATY, A. S., AHRENS, S., ANDERSON, W. F. & HAUSER, A. R. (2010) Role of the membrane localization domain of the *Pseudomonas aeruginosa* effector protein ExoU in cytotoxicity. *Infect Immun,* 78, 3346-57.
VERNOZYROZAND, C. & RAYGUENIOT, S. (1997) *Escherichia coli* O157:H7: Clinic, pathogenesis, epidemiology and prevention of the foodborne infections. *Rev Med Vet,* 148, 89-98.
VIEGAS, S. C., PFEIFFER, V., SITTKA, A., SILVA, I. J., VOGEL, J. & ARRAIANO, C. M. (2007) Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res,* 35, 7651-64.

VIMR, E. R. & STEENBERGEN, S. M. (2006) Mobile contingency locus controlling *Escherichia coli* K1 polysialic acid capsule acetylation. *Mol Microbiol,* 60, 828-37.
VINGADASSALOM, D., KAZLAUSKAS, A., SKEHAN, B., CHENG, H. C., MAGOUN, L., ROBBINS, D., ROSEN, M. K., SAKSELA, K. & LEONG, J. M. (2009) Insulin receptor tyrosine kinase substrate links the *Escherichia coli* O157:H7 actin assembly effectors Tir and EspF(U) during pedestal formation. *Proc Natl Acad Sci U S A,* 106, 6754-9.
VOGEL, J. (2009) A rough guide to the non-coding RNA world of *Salmonella*. *Mol Microbiol,* 71, 1-11.
VOGEL, J. & PAPENFORT, K. (2006) Small non-coding RNAs and the bacterial outer membrane. *Curr Opin Microbiol,* 9, 605-11.
VOGEL, J. & SHARMA, C. M. (2005) How to find small non-coding RNAs in bacteria. *Biol Chem,* 386, 1219-38.
VOGEL, J. & WAGNER, E. G. (2007) Target identification of small noncoding RNAs in bacteria. *Curr Opin Microbiol,* 10, 262-70.
VYTVYTSKA, O., JAKOBSEN, J. S., BALCUNAITE, G., ANDERSEN, J. S., BACCARINI, M. & VON GABAIN, A. (1998) Host factor I, Hfq, binds to *Escherichia coli* ompA mRNA in a growth rate dependent fashion and regulates its stability. *Proc Natl Acad Sci U S A,* 95, 14118-14123.
WACHTER, C., BEINKE, C., MATTES, M. & SCHMIDT, M. A. (1999) Insertion of EspD into epithelial target cell membranes by infecting enteropathogenic *Escherichia coli*. *Mol Microbiol,* 31, 1695-1707.
WAGNER, S., SORG, I., DEGIACOMI, M., JOURNET, L., DAL PERARO, M. & CORNELIS, G. R. (2009) The helical content of the YscP molecular ruler determines the length of the *Yersinia* injectisome. *Mol Microbiol,* 71, 692-701.
WAINWRIGHT, L. A. & KAPER, J. B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Mol Microbiol,* 27, 1247-1260.
WALKER-SMITH, J. A. (1996) Evolution of concept of infective origin of infantile diarrhoea from Ballard to Bray (1887-1945). *J Infect,* 33, 213-8.
WANG, B., MO, Z. L., MAO, Y. X., ZOU, Y. X., XIAO, P., LI, J., YANG, J. Y., YE, X. H., LEUNG, K. Y. & ZHANG, P. J. (2009) Investigation of EscA as a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon component EseC. *Microbiology,* 155, 1260-71.
WANG, D., ROE, A. J., MCATEER, S., SHIPSTON, M. J. & GALLY, D. L. (2008) Hierarchal type III secretion of translocators and effectors from *Escherichia coli* O157:H7 requires the carboxy terminus of SepL that binds to Tir. *Mol Microbiol,* 69, 1499-512.
WARAWA, J., FINLAY, B. B. & KENNY, B. (1999) Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. *Infect Immun,* 67, 5538-40.
WATANABE, Y., OZASA, K., MERMIN, J. H., GRIFFIN, P. M., MASUDA, K., IMASHUKU, S. & SAWADA, T. (1999) Factory outbreak of *Escherichia coli* O157:H7 infection in Japan. *Emerg Infect Dis,* 5, 424-8.
WATARAI, M., TOBE, T., YOSHIKAWA, M. & SASAKAWA, C. (1995) Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness. *EMBO J,* 14, 2461-70.

WATTIAU, P., BERNIER, B., DESLEE, P., MICHIELS, T. & CORNELIS, G. R. (1994) Individual chaperones required for Yop secretion by *Yersinia*. *Proc Natl Acad Sci U S A,* 91, 10493-7.
WATTS, K. M. & HUNSTAD, D. A. (2008) Components of SurA required for outer membrane biogenesis in uropathogenic *Escherichia coli*. *PLoS One,* 3, e3359.
WEBSTER, D., COWDEN, J. & LOCKING, M. (2007) An outbreak of *Escherichia coli* O157 in Aberdeen, Scotland, September 2007. *Euro Surveill,* 12, E070927 1.
WEI, B. L., BRUN-ZINKERNAGEL, A. M., SIMECKA, J. W., PRUSS, B. M., BABITZKE, P. & ROMEO, T. (2001) Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol Microbiol,* 40, 245-56.
WEI, Z. M., LABY, R. J., ZUMOFF, C. H., BAUER, D. W., HE, S. Y., COLLMER, A. & BEER, S. V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science,* 257, 85-8.
WEISS, S. M., LADWEIN, M., SCHMIDT, D., EHINGER, J., LOMMEL, S., STADING, K., BEUTLING, U., DISANZA, A., FRANK, R., JANSCH, L., SCITA, G., GUNZER, F., ROTTNER, K. & STRADAL, T. E. (2009) IRSp53 links the enterohemorrhagic *Escherichia coli* effectors Tir and EspFU for actin pedestal formation. *Cell Host Microbe,* 5, 244-58.
WENDEL, A. M., JOHNSON, D. H., SHARAPOV, U., GRANT, J., ARCHER, J. R., MONSON, T., KOSCHMANN, C. & DAVIS, J. P. (2009) Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. *Clin Infect Dis,* 48, 1079-86.
WHALE, A. D., GARMENDIA, J., GOMES, T. A. & FRANKEL, G. (2006) A novel category of enteropathogenic *Escherichia coli* simultaneously utilizes the Nck and TccP pathways to induce actin remodelling. *Cell Microbiol,* 8, 999-1008.
WHITE-ZIEGLER, C. A. & DAVIS, T. R. (2009) Genome-wide identification of H-NS-controlled, temperature-regulated genes in *Escherichia coli* K-12. *J Bacteriol,* 191, 1106-10.
WIESER, A., ROMANN, E., MAGISTRO, G., HOFFMANN, C., NORENBERG, D., WEINERT, K. & SCHUBERT, S. (2010) A multiepitope subunit vaccine conveys protection against extraintestinal pathogenic Escherichia coli in mice. Infect Immun, 78, 3432-42.
WILES, T. J., KULESUS, R. R. & MULVEY, M. A. (2008) Origins and virulence mechanisms of uropathogenic *Escherichia coli*. *Exp Mol Pathol,* 85, 11-9.
WILHARM, G., DITTMANN, S., SCHMID, A. & HEESEMANN, J. (2007) On the role of specific chaperones, the specific ATPase, and the proton motive force in type III secretion. *Int J Med Microbiol,* 297, 27-36.
WILHARM, G., LEHMANN, V., KRAUSS, K., LEHNERT, B., RICHTER, S., RUCKDESCHEL, K., HEESEMANN, J. & TRULZSCH, K. (2004) *Yersinia enterocolitica* type III secretion depends on the proton motive force but not on the flagellar motor components MotA and MotB. *Infect Immun,* 72, 4004-9.
WILSON, R. K., SHAW, R. K., DANIELL, S., KNUTTON, S. & FRANKEL, G. (2001) Role of EscF, a putative needle complex protein, in the type III

protein translocation system of enteropathogenic *Escherichia coli*. *Cell Microbiol,* 3, 753-62.
WISE, J. (2009) Outbreak of E coli O157 is linked to Surrey open farm. *BMJ,* 339, b3795.
WOLD, A. E. & ADLERBERTH, I. (2000) Breast feeding and the intestinal microflora of the infant--implications for protection against infectious diseases. *Adv Exp Med Biol,* 478, 77-93.
WONG, K. R. & BUCKLEY, J. T. (1989) Proton motive force involved in protein transport across the outer membrane of *Aeromonas salmonicida*. *Science,* 246, 654-6.
WULLT, B., BERGSTEN, G., CONNELL, H., ROLLANO, P., GEBRETSADIK, N., HULL, R. & SVANBORG, C. (2000) P fimbriae enhance the early establishment of *Escherichia coli* in the human urinary tract. *Mol Microbiol,* 38, 456-64.
XIE, H. X., YU, H. B., ZHENG, J., NIE, P., FOSTER, L. J., MOK, Y. K., FINLAY, B. B. & LEUNG, K. Y. EseG, an effector of the type III secretion system of *Edwardsiella tarda* triggers microtubule destabilization. *Infect Immun*.
XIE, Y., KIM, K. J. & KIM, K. S. (2004) Current concepts on *Escherichia coli* K1 translocation of the blood-brain barrier. *FEMS Immunol Med Microbiol,* 42, 271-9.
YAHR, T. L. & WOLFGANG, M. C. (2006) Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol,* 62, 631-40.
YANG, H., SHAN, Z., KIM, J., WU, W., LIAN, W., ZENG, L., XING, L. & JIN, S. (2007) Regulatory role of PopN and its interacting partners in type III secretion of *Pseudomonas aeruginosa*. *J Bacteriol,* 189, 2599-609.
YANG, J., JAIN, C. & SCHESSER, K. (2008) RNase E regulates the *Yersinia* type 3 secretion system. *J Bacteriol,* 190, 3774-8.
YANG, Z., KIM, J., ZHANG, C., ZHANG, M., NIETFELDT, J., SOUTHWARD, C. M., SURETTE, M. G., KACHMAN, S. D. & BENSON, A. K. (2009) Genomic instability in regions adjacent to a highly conserved pch prophage in *Escherichia coli* O157:H7 generates diversity in expression patterns of the LEE pathogenicity island. *J Bacteriol,* 191, 3553-68.
YEATS, C. & BATEMAN, A. (2003) The BON domain: a putative membrane-binding domain. *Trends Biochem Sci,* 28, 352-5.
YIP, C. K., FINLAY, B. B. & STRYNADKA, N. C. (2005a) Structural characterization of a type III secretion system filament protein in complex with its chaperone. *Nat Struct Mol Biol,* 12, 75-81.
YIP, C. K., KIMBROUGH, T. G., FELISE, H. B., VUCKOVIC, M., THOMAS, N. A., PFUETZNER, R. A., FREY, E. A., FINLAY, B. B., MILLER, S. I. & STRYNADKA, N. C. (2005b) Structural characterization of the molecular platform for type III secretion system assembly. *Nature,* 435, 702-7.
YIP, C. K. & STRYNADKA, N. C. (2006) New structural insights into the bacterial type III secretion system. *Trends Biochem Sci,* 31, 223-30.
YOON, Y., MUKHERJEE, A., BELK, K. E., SCANGA, J. A., SMITH, G. C. & SOFOS, J. N. (2009) Effect of tenderizers combined with organic acids on *Escherichia coli* O157:H7 thermal resistance in non-intact beef. *Int J Food Microbiol,* 133, 78-85.
YOSHIOKA, K., YAGI, K. & MORIGUCHI, N. (1999) Clinical features and treatment of children with hemolytic uremic syndrome caused by

enterohemorrhagic *Escherichia coli* O157:H7 infection: experience of an outbreak in Sakai City, 1996. *Pediatr Int,* 41, 223-7.
YOUNG, G. M., SCHMIEL, D. H. & MILLER, V. L. (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci U S A,* 96, 6456-61.
YOUNIS, R., BINGLE, L. E., ROLLAUER, S., MUNERA, D., BUSBY, S. J., JOHNSON, S., DEANE, J. E., LEA, S. M., FRANKEL, G. & PALLEN, M. J. (2010) SepL resembles an aberrant effector in binding to a class 1 type-III secretion chaperone and carrying an N-terminal secretion signal. *J Bacteriol*. 192, 6093-8.
YU, X. J., MCGOURTY, K., LIU, M., UNSWORTH, K. E. & HOLDEN, D. W. (2010) pH sensing by intracellular *Salmonella* induces effector translocation. *Science,* 328, 1040-3.
YU, X. J., RUIZ-ALBERT, J., UNSWORTH, K. E., GARVIS, S., LIU, M. & HOLDEN, D. W. (2002) SpiC is required for secretion of *Salmonella* Pathogenicity Island 2 type III secretion system proteins. *Cell Microbiol,* 4, 531-40.
ZARIVACH, R., DENG, W., VUCKOVIC, M., FELISE, H. B., NGUYEN, H. V., MILLER, S. I., FINLAY, B. B. & STRYNADKA, N. C. (2008) Structural analysis of the essential self-cleaving type III secretion proteins EscU and SpaS. *Nature,* 453, 124-7.
ZARIVACH, R., VUCKOVIC, M., DENG, W., FINLAY, B. B. & STRYNADKA, N. C. (2007) Structural analysis of a prototypical ATPase from the type III secretion system. *Nat Struct Mol Biol,* 14, 131-7.

Appendix 1

A1.1 Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit

1. 1–5 ml overnight cultures of *E. coli* in LB medium were harvested by centrifugation at 13000xg for 5 mins.
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
7. Centrifuge for 30–60 s. Discard the flow-through.
8. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
9. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
10. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
11. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

A1.2 QIAquick PCR Purification Kit Protocol using a microcentrifuge

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add

30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

A1.3 QIAquick Gel Extraction Kit Protocol using a microcentrifuge

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.
14. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

A1.4 Polymerase Chain Reaction (PCR)

1. Setup a 50 µl PCR reaction in a DNase free PCR tube in the following order: ①5 µl 10x PCR reaction buffer(Containing Mg^{2+}); ②2.5 µl foreward primer (10 µM); ③2.5 µl reverse primer (10 µM); ④2 µl dNTP mix (10 mM each); ⑤0.2 µl Taq polymerase (5 units/µl); ⑥ 32.8 µl dH2O; ⑦1 µl DNA template (10ng-500ng).

2. Setup 3-steps thermal cycles as following example:
   ①1 cycle: 95°C (denature) for 4 mins;
   ②20-30 cycles: 95°C (denature) for 45 seconds; 55°C (anneling) for 45 seconds; 72°C (extension) for several mins (1 minute/kb of product length).
   ③72°C for 10 mins (final extension), then keep at 4°C.

A1.5 Purification of total RNA (Invitrogen ChargeSwitch® Total RNA Cell Kits)

A1.5.1 Preparing Bacterial Lysates

1. Harvest 1-10 ml bacterial culture by centrifugation. Completely remove the growth medium.
2. Wash the cells with 1X PBS. Centrifuge the cells and completely remove the PBS from the cell pellet.
3. Resuspend the cells in 500 µl Lysis Mix. Pipet up and down thoroughly (up to 15 times) until the pellet is broken up and the lysate is no longer viscous.
4. Incubate at 60°C for 15 minutes.
5. After incubation, vortex the lysate briefly to mix. Cool the samples for 1 minute on ice.

A1.5.2 Binding RNA

1. Thoroughly vortex the tube containing the ChargeSwitch® Magnetic Beads to fully resuspend the beads in the storage buffer.
2. Add 100 µl of ChargeSwitch® Magnetic Beads to the lysate.
3. Add 200 µl Binding Buffer (B9) to the samples and mix by pipetting up and down gently 5 times using a 1 ml adjustable pipette tip set to 700 µl to mix the sample without forming bubbles.
4. Incubate at room temperature for 1 minute to allow the RNA to bind to the beads.
5. Place the sample on the MagnaRack™ until the beads have formed a tight pellet and the supernatant is clear.
6. Without removing the tube from the magnet, carefully aspirate and discard the supernatant without disturbing the pellet of beads by angling the pipette such that the tip is pointed away from the pellet.

A1.5.3 DNase I Treatment

1. Remove the tube containing the pelleted magnetic beads from the magnet. There should be no supernatant in the tube.
2. Add 500 µl Wash Buffer (W14) to the tube and pipet up and down gently 5 times to resuspend the magnetic beads using a 1 ml pipette tip set to 700 µl to mix the sample without forming bubbles.

3. Place the sample on the magnet until the beads have formed a tight pellet and the supernatant is clear.
4. Without removing the tube from the magnet, carefully aspirate and discard the supernatant without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet. Remove the tube from the magnet.
5. Add 250 µl of the prepared DNase I Mix. Resuspend the magnetic beads by pipeting up and down gently 5 times using a 1 ml pipette tip set to 200 µl to mix the sample without forming bubbles or vortex at low speed to avoid any solution from collecting in the cap of the tube.
6. Incubate at room temperature for 10 minutes.
7. Add 80 µl Binding Buffer (B9) to the sample and pipet up and down gently 5 times using a 1 ml pipette tip set to 200 µl to mix the sample without forming bubbles or vortex at low speed to resuspend the magnetic beads.
8. Incubate at room temperature for 1 minute.
9. Place the sample on the magnet until the beads have formed a tight pellet and the supernatant is clear.
10. Without removing the tube from the magnet, carefully aspirate and discard the supernatant without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet.

A1.5.4 Washing RNA

1. Remove the tube containing the pelleted magnetic beads from the magnet. There should be no supernatant in the tube.
2. Add 750 µl Wash Buffer (W13) to the tube. Resuspend the magnetic beads by pipeting up and down gently at least 5 times using a 1 ml pipette tip set to 700 µl to mix the sample without forming bubbles or vortex at low speed to avoid any solution from collecting in the tube cap.
3. Place the sample on the magnet until the beads have formed a tight pellet and the supernatant is clear.
4. Without removing the tube from the magnet, carefully aspirate and discard the supernatant without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet. Remove the tube from the magnet.
5. Add 750 µl Wash Buffer (W14) or 500 µl. Resuspend the magnetic beads by pipeting up and down gently 3 times to using a 1 ml adjustable pipette tip set to 700 µl to mix the sample without forming bubbles or vortex at low speed.
6. Place the sample on the magnet until the beads have formed a tight pellet and the supernatant is clear.
7. Without removing the tube from the magnet, carefully aspirate and discard the supernatant without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet.

A1.5.5 Eluting RNA

1. Remove the tube containing the pelleted magnetic beads from the magnet. There should be no supernatant in the tube
2. Add 150 µl Elution Buffer (E7) to the tube. Resuspend the magnetic beads by pipeting up and down gently 10 times using a pipette tip set to 120 µl to mix the sample or vortex at low speed.
3. Incubate at room temperature for 5 minutes.
4. Place the sample on the magnet until the beads have formed a tight pellet and the supernatant is clear. Without removing the tube from the magnet, carefully transfer the supernatant containing the RNA to a sterile microcentrifuge tube without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet.
5. Discard the used magnetic beads. Do not reuse the magnetic beads.
6. Store the purified total RNA at -80°C or use the RNA for the desired downstream application.

A1.6 NorthernMax® Kit Instruction Manual

(Before Start, Use the RNaseZap® Solution provided with the kit to remove any contaminating.)

A1.6.1 Preparation of Gel

1. Melt 1 g agarose in 90 mL RNase-free water for every 100 mL of gel.
2. Add 10 mL 10X Denaturing Gel Buffer per 100 mL of gel.
3. Pour the gel to about 0.6 cm in thickness and allow the gel to solidify at RT or at 4°C, remove the comb.
4. Set up the electrophoresis chamber.

A1.6.2 Preparation of Sample RNA

1. Mix sample RNA with 3 volumes Formaldehyde Load Dye and incubate the samples 15 min at 65°C.
2. Load the RNA samples into the wells of the gel and Run the gel at ~5 V/cm.
3. (optional) Examine the gel with UV light, and photograph it.

A1.6.3 Transfer of RNA to the Membrane

1. Prepare blotting materials and assemble the transfer materials as shown in Fig.A2.1.
2. Transfer for 15–20 min per mm of gel thickness.
3. Disassemble the transfer setup and crosslink the RNA (UV 290nm/60 s).

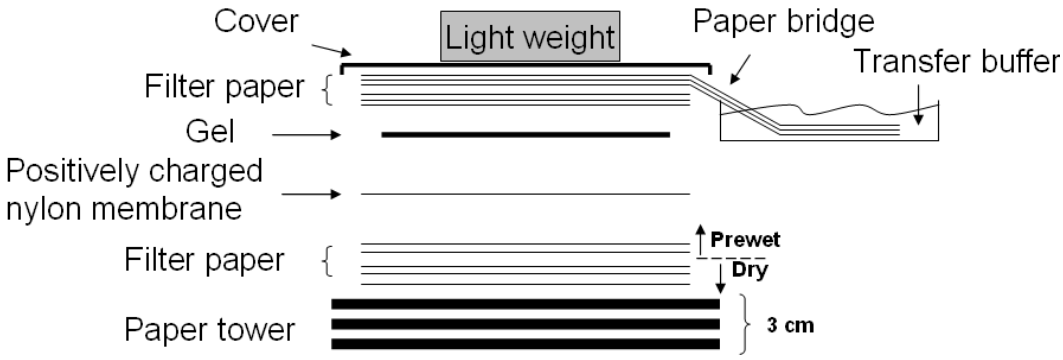


Fig. A1.1 Schematic diagram of Downward Transfer Assembly

A1.6.4 Prehybridization and Hybridization

1. Preheat ULTRAhyb to 68°C and prehybridize ⩾30 min at 42°C (10 mL preheated ULTRAhyb Buffer per 100 cm^2 of membrane).
2. Add a DNA probe to the prehybridized blot and hybridize 2 hr to overnight.

A1.6.5 Washing and Exposure to Film

1. 2X 5 min, room temperature washes with Low Stringency Wash Solution1 (Agitation).
2. 2X 15 min, 42°C washes with High Stringency Wash Solution 2 (Agitation).
3. Seal radiolabeled blots in plastic and expose X-ray film

A1.7 QuikChange Site-Directed Mutagenesis (Stratagene)

1. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide primers prior to use in the following steps. (Mutagenic primers can be designed using web-based QuikChange Primer Design Program available online at http://www.stratagene.com/qcprimerdesign)
2. Set up reactions as suggested in User's Manual (5 µl of 10× reaction buffer; 2 µl (20 ng) of dsDNA template; 2.5 µl (125 ng) of each oligonucleotide primer; 1 µl of dNTP mix; 36 µl ddH_2O) and start thermal cycles as shown in table below.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C	30 seconds
		55°C	1 minutes
		68°C	7 minutes

3. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to ≤37°C.
4. Add 1 µl of the *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction and gently mix each reaction mixture by pipetting the solution up and down. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental dsDNA.
5. Transfer 1 µl of the *Dpn* I-treated DNA from each sample reaction to separate aliquots of the XL1-Blue supercompetent cells. Heat shock the transformation reactions for 45 seconds at 42°C and then place the reactions on ice for 2 minutes. Add 0.5 ml of NZY+ broth preheated to 42°C and incubate the transformation reactions at 37°C for 1 hour with shaking at 200 rpm.
6. Plate 200 µl of each transformation reaction on LB(chloramphenicol) agar plate and Incubate the transformation plates at 28°C for >16 hours.
7. Pick 8 colonies and extract DNA plasmid using the QIAprep Spin Miniprep Kit.
8. DNA plasmid sequence was then examined by DNA sequencing.

Appendix 2

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Hierarchal type III secretion of translocators and effectors from *Escherichia coli* O157:H7 requires the carboxy terminus of SepL that binds to Tir

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Summary

Type III secretion (T3S) from enteric bacteria is a co-ordinated process with a hierarchy of secreted proteins. In enteropathogenic and enterohaemorrhagic *Escherichia coli*, SepL and SepD are essential for translocator but not effector protein export, but how they function to control this differential secretion is not known. This study has focused on the different activities of SepL including membrane localization, SepD binding, EspD export and Tir secretion regulation. Analyses of SepL truncates demonstrated that the different functions associated with SepL can be separated. In particular, SepL with a deletion of 11 amino acids from the C-terminus was able to localize to the bacterial membrane, export translocon proteins but not regulate Tir or other effector protein secretion. From the repertoire of effector proteins only Tir was shown to bind directly to full-length SepL and the C-terminal 48 amino acids of SepL was sufficient to interact with Tir. By synchronizing induction of T3S, it was evident that the Tir-binding capacity of SepL is important to delay the release of effector proteins while the EspADB translocon is secreted. The interaction between Tir and SepL is therefore a critical step that controls the timing of T3S in attaching and effacing pathogens.

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is the main EHEC serotype associated with outbreaks of gastrointestinal disease in North America, parts of Europe and Japan. It is an important pathogen that can be life-threatening particularly in the young and the elderly. EHEC strains express a type III secretion (T3S) system encoded on the locus of enterocyte effacement (LEE) pathogenicity island (Jerse *et al.*, 1990; McDaniel *et al.*, 1995). The main phenotype associated with EHEC O157 T3S is the formation of attaching and effacing (A/E) lesions (Knutton *et al.*, 1989; 1998). A/E lesions require the injection of the translocated intimin receptor (Tir) into the enterocyte (Kenny *et al.*, 1997; Deibel *et al.*, 1998) and the cytoskeleton rearrangements associated with A/E lesions require Tir and EspFu/TccP (Campellone *et al.*, 2004; Garmendia *et al.*, 2004) that act through N-WASP activation of the Arp2/3 complex leading to alpha-actinin polymerization (Garmendia *et al.*, 2005).

The T3S system is evident as a needle-like projection on the surface of the bacterium. Its predicted structure is considered to have the proteins EscD (Pas), R, S, T, U and V in the inner membrane joined to an outer membrane ring of EscC, with EscF forming a needle-like structure at the base of the EspA filament (Daniell *et al.*, 2001; Yip *et al.*, 2005). The export of Tir and other effector proteins occurs through the hollow filament, with the timing and regulation of translocation potentially driven by chaperone proteins (Wainwright and Kaper, 1998; Elliott *et al.*, 1999; Neves *et al.*, 2003). The filament is considered to link to a pore in the epithelial cell membrane that is composed of the bacterial protein EspD, and possibly EspB (Wachter *et al.*, 1999). Export of effector proteins into the host cell is therefore dependent on the initial export and assembly of EspADB. The conduit that these three proteins form has been referred to as a 'translocon' and the proteins as 'translocators' (Deng *et al.*, 2005).

The LEE pathogenicity island contains at least 41 genes in five main operons. *espADB* are expressed from the LEE4 operon and are preceded in this operon by

sepL. *sepL* encodes a protein composed of 351 amino acids (aa) with a predicted molecular weight of 39.95 kDa (Kresse *et al*., 2000). Like many genes in the LEE it is highly conserved between EHEC and enteropathogenic *E. coli* (EPEC) strains (93.7–94.3% identity). SepL is central to a switch that occurs between the export of EspADB and the export of effector proteins. Original research by Kresse *et al*. (2000) indicated that SepL was associated predominately with the bacterial membrane and in particular the outer membrane. Research using EPEC O127 has demonstrated that the majority of SepL is either in the cytoplasm (O'Connell *et al*., 2004) or in both the cytoplasm and the inner membrane (Deng *et al*., 2005).

How SepL works to govern the switch between translocon and effector protein secretion is not known, but both yeast two-hybrid and *in vitro* studies have demonstrated that SepL interacts directly with SepD, a protein encoded on LEE2 (Creasey *et al*., 2003; O'Connell *et al*., 2004). A deletion of *sepD* has a similar phenotype to a *sepL* mutant, in that the export of translocators but not effectors is prevented (Deng *et al*., 2004). In fact, deletion of either *sepL* or *sepD* leads to an increase in the levels of secreted Tir and other effector proteins and this increased effector protein secretion is not considered to be controlled at the transcriptional level (Deng *et al*., 2004; 2005). A recent hypothesis is that SepL and SepD act as a gate to allow translocator export, but that this gate dissociates or changes in response to a drop in calcium levels following the opening of a conduit to the host cell, allowing effectors to be exported (Deng *et al*., 2005). However, there is no evidence that SepL or SepD interacts directly with the translocon proteins EspA, D or B. The majority of SepL (267 aa) shares some homology with YopN and its carboxy terminus (83 aa) homology with TyeA, both from *Yersinia* spp. (Pallen *et al*., 2005). As TyeA controls the secretion of specific effector proteins in *Yersinia* spp. (Cheng and Schneewind, 2000; Day *et al*., 2003; Sundberg and Forsberg, 2003) it was decided to investigate full-length and carboxy-terminal truncates of SepL in terms of the known activities of SepL including localization, SepD binding, translocon export and Tir secretion control.

Results

SepL is homologous to a combination of YopN and TyeA from *Yersinia* spp. (Pallen *et al*., 2005; Fig. 8). The carboxy terminus of SepL (final 83 aa) is homologous with TyeA and so carboxy-terminal truncates of SepL fused to eGFP were tested to determine if these deletions can separate the different functions of SepL. The functions analysed were the capacity to: (i) localize to the bacterial membrane, (ii) bind to SepD, (iii) restore EspD secretion in *sepL* mutants and (iv) reduce Tir secretion in *sepL* mutants. Five truncated proteins were initially constructed as illustrated in Fig. 1A.

Membrane localization and the role of SepD

Previous research has demonstrated that SepL in EHEC O157 can be membrane-associated and can also bind to SepD (Kresse *et al*., 2000; Creasey *et al*., 2003; O'Connell *et al*., 2004). The different-length fusion constructs were examined for their presence in bacterial membrane-containing fractions. The full-length SepL–eGFP construct was present in membrane-containing preparations of bacteria prepared from T3S-permissive conditions while this was not the case for eGFP alone, which was only detectable in the cytoplasmic fraction (Fig. 1B). Deletion of either 11 aa or 61 aa from the carboxyl end of SepL (creating 340 aa and 290 aa proteins fused to eGFP) still allowed detection in the membrane-containing fractions, although this distribution was prevented by any further truncation with detection only in the cytoplasmic fraction. As controls, the distributions of OmpA and GroEL in the same samples were as expected (OmpA > 90% in the membrane-containing fraction; GroEL > 99% in the cytoplasmic fraction) (Fig. 1B). Direct imaging of individual bacteria containing the full-length SepL–eGFP clearly showed a higher concentration of fluorescence localized to the periphery of the bacteria (Fig. 1C). This was not the case for bacteria expressing just eGFP (Fig. 1C). The biochemical and imaging data indicated that SepL localizes to the bacterial membrane and this association does not require the carboxy-terminal 61 aa of SepL.

We next wanted to determine what effect *sepD* deletion would have on the localization of SepL. *sepD* was deleted by allelic exchange and the construct confirmed by complementation with *sepD* on a plasmid (pDW20, data not shown). Localization of SepL–eGFP was examined using a combination of Western blotting and single cell imaging (Figs 2A and B and 1C). The proportion of the full-length SepL–eGFP hybrid protein that was in the membrane-containing fraction was reduced in a *sepD* background (Fig. 2A). In addition, in this background, the localization of SepL–eGFP was clearly different in the bacteria. The distribution now appeared asymmetric (Fig. 2B), unlike the distribution of eGFP alone, indicating that in the absence of SepD, SepL may associate with another cellular protein that exhibits this asymmetric distribution. The different SepL truncates fused to eGFP were then assessed for their capacity to bind to purified GST–SepD attached to a glutathione-sepharose 4B column. Full-length SepL as well as the 340 aa and 290 aa fusions bound to SepD but further truncation from the carboxy terminus prevented the interaction (Fig. 3).

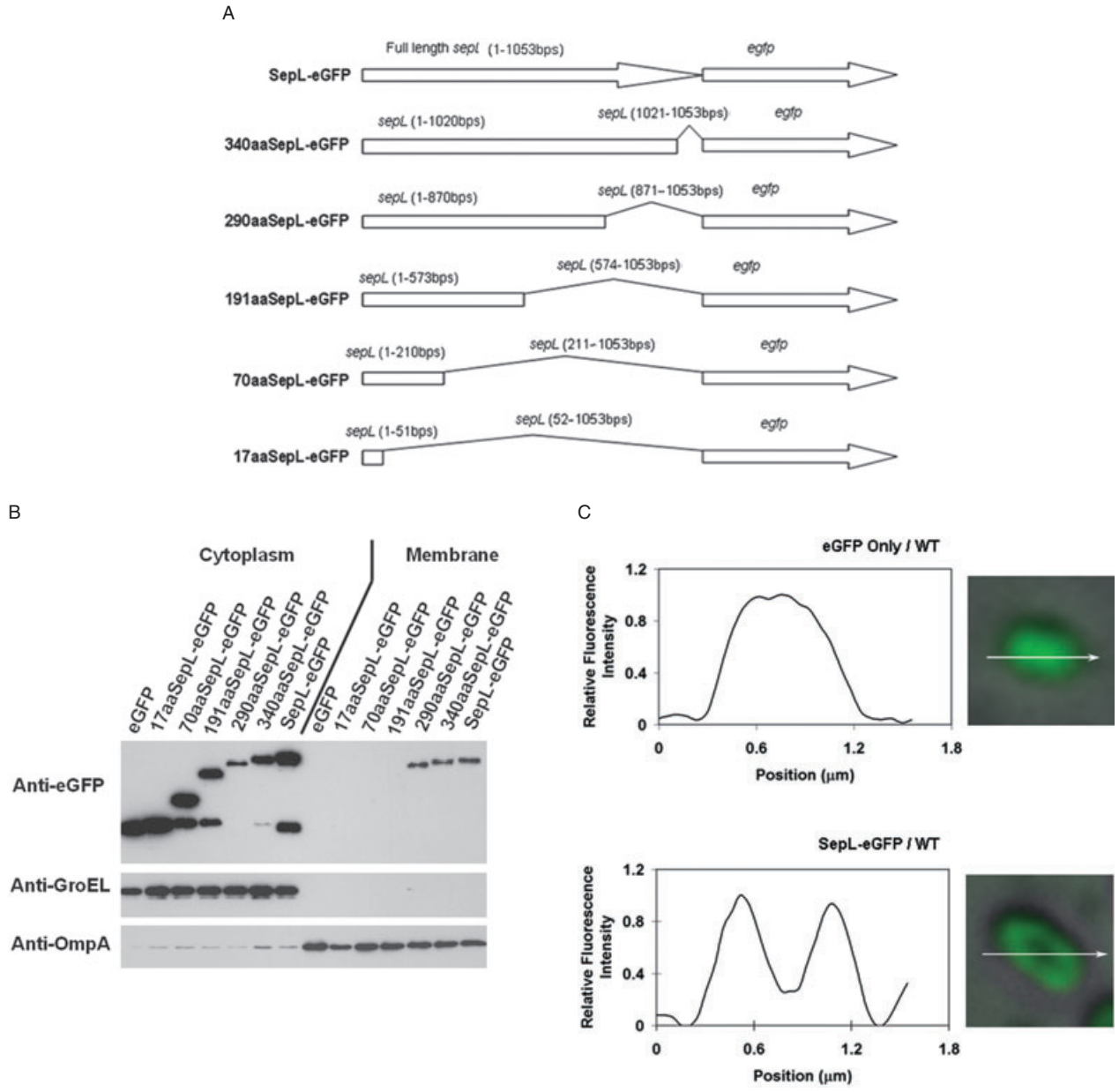

Fig. 1. Analysis of SepL–eGFP localization.
A. Figure of the SepL truncates fused to eGFP that were used in the study. The full-length, 340 aa, 290 aa and 267 aa SepL regions were also generated as carboxy-terminal 6× His-tagged constructs.
B. Membrane localization of SepL truncates fused to eGFP. Western blotting was used to detect eGFP as described in *Experimental procedures*. Integrity of the fractions was confirmed with anti-GroEL and anti-OmpA antibodies.
C. Fluorescence and phase contrast image overlays demonstrating localization of eGFP and SepL–eGFP in a single bacterium. Fluorescence levels were measured along a transect drawn through individual cells. The examples shown demonstrate the peripheral localization of SepL–eGFP but not eGFP alone.

The SepL truncates that localized to the membrane are the same as those that bound to SepD. Given that deletion of *sepD* also reduced membrane association of SepL and altered its cellular distribution, it is evident that SepD is probably required for SepL localization to the bacterial membrane.

Complementation of translocon (EspD) export in a sepL mutant

SepL is required for translocon but not effector protein export and translocon export can be complemented in a *sepL* mutant by supplying *sepL in trans* (Kresse *et al.*,

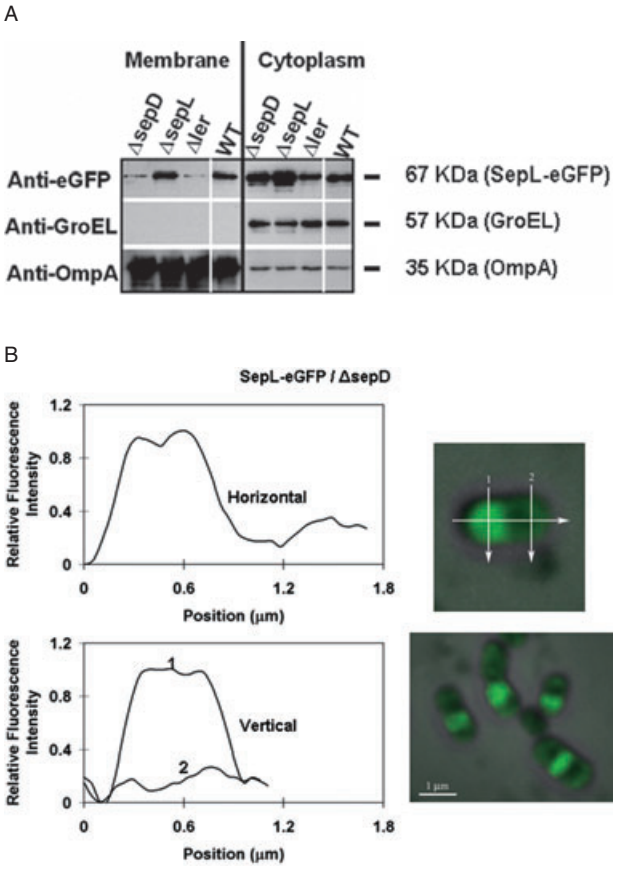

Fig. 2. SepL–eGFP localization in different EHEC O157 genetic backgrounds.
A. Western blot detection of eGFP in membrane and cytoplasmic fractions of the described EHEC O157 strains expressing SepL–eGFP (pDW6). Western blotting was carried out as described in *Experimental procedures*.
B. Localization of SepL–eGFP in a *sepD* mutant. Fluorescence intensities across a representative bacterium expressing the SepL–eGFP fusion are shown. The asymmetric distribution of the SepL–eGFP fusion in a *sepD* mutant background (ZAP1144) is apparent when compared with the distribution in the wild-type background (Fig. 1C).

2000). This was also the case in this study in which the full-length SepL–eGFP fusion could complement a complete *sepL* deletion mutant for EspD secretion (Fig. 4A). In our hands this complementation never achieved the EspD secretion levels of the wild-type strain and this was also the case for complementation with *sepL* alone, pDW24 (data not shown). Analysis of the SepL truncates in the *sepL* deletion strain indicated that only the full-length protein and the 11 aa carboxy-terminal deletion were able to export EspD. Deletion of 11 aa from SepL partially complemented the *sepL* deletion for EspD secretion (Fig. 4A). It was interesting to note that a deletion of 61 aa (leaving a 290 aa SepL derivative) failed to export EspD despite membrane localization and SepD binding activity.

Our ongoing work indicates that the failure to completely complement a full *sepL* deletion is due to changes in the LEE4 transcript (data not shown). To verify the phenotypes of the SepL truncates in a *sepL*-mutated background, a frameshift mutation was constructed in *sepL* (ZAP1211) that will have less impact on the structure of the LEE4 transcript. In this background, EspD secretion could be complemented completely by *sepL in trans* (Fig. 4D). The 11 aa carboxy-terminal deletion in this background was still able to restore some EspD secretion, although at markedly reduced levels compared with the full-length complement (Fig. 4D).

Regulation of effector protein secretion

A *sepL* mutant is characterized by high levels of Tir secretion (Kresse *et al*., 2000 and Fig. 4B) and of other effector proteins including NleA (Deng *et al*., 2004). To investigate the function of the different SepL truncate fusions in this capacity, the levels of Tir secreted were determined by Western blotting in the *sepL* mutants transformed with the different SepL constructs. Of note was that only the full-length SepL construct was able to lower Tir secretion levels to those demonstrated for the wild-type strain (Fig. 4B). To confirm this result and to rule out any impact of eGFP on the phenotypes, four His-tagged SepL constructs were also tested. These were: (i) full-length SepL, (ii) a protein with the first 340 aa of SepL but deleted for the carboxy-terminal 11 aa, (iii) the first 290 aa of SepL but deleted for the carboxy-terminal 61 aa and (iv) the first 267 aa of SepL but deleted for the carboxy-terminal 84 aa. These variants had exactly the same phenotypes as the respective eGFP fusions in the *sepL* deletion background. For example, analysis of the general secretion profiles indicated that the 11 aa carboxy-terminal SepL truncate failed to control secretion of Tir, whereas

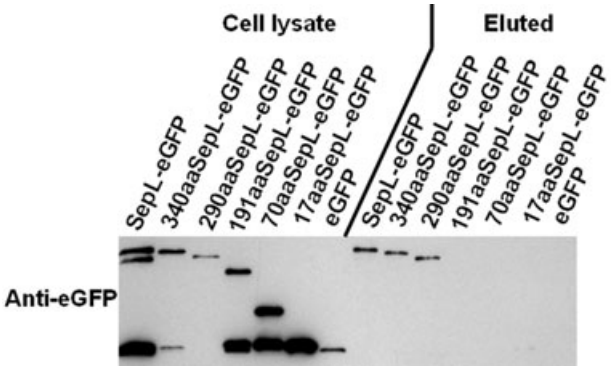

Fig. 3. Capacity of SepL truncates to bind to SepD. Each of the SepL–eGFP constructs was tested for the capacity to bind to immobilized GST–SepD. Following elution, the SepL–eGFP truncates were detected by Western blotting as described in *Experimental procedures*.

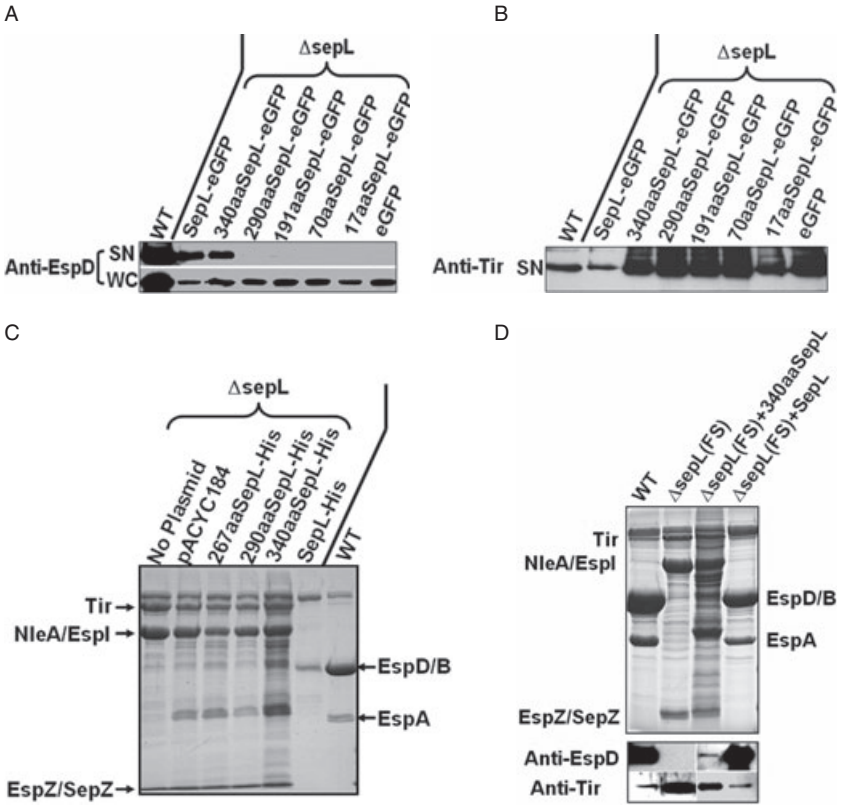

Fig. 4. Analysis of protein secretion in *E. coli* O157 (Δ*sepL*) expressing different SepL truncates.
A. The first two panels show EspD levels in the supernatant (SN) and whole-cell fractions (WC) when the different SepL–eGFP constructs were used to complement a *sepL* mutant (ZAP1143).
B. The panel shows detection of secreted Tir by Western blotting from the samples labelled in (A).
C. Analysis of bacterial supernatants from a *sepL* mutant complemented with the His-tagged SepL constructs described in the Fig. 1 and the text. The wild-type (ZAP193) supernatant profile is also shown for comparison. These experiments confirm the results obtained with the SepL–eGFP constructs in (A) and (B) and demonstrate that the carboxy-terminal 11 aa of SepL are required to limit the secretion of effector proteins in addition to Tir.
D. Colloidal blue staining of secreted proteins from *E. coli* O157 engineered to contain a frameshift mutation in *sepL* (ZAP1211) and then complemented with full-length SepL (pDW48) and the C-terminal 11 aa deletion of SepL (pDW47). Western blotting for EspD and Tir for these strains is also shown. Preparation of protein samples and detection of Tir and EspD by Western blotting were as described in *Experimental procedures*.

complementation with full-length SepL could (Fig. 4B and C). Moreover, it was apparent that this regulation also applied to other T3S effectors with sizes equivalent to those of NleA and EspZ, as complementation with full-length SepL restored normal secretion levels in a *sepL* mutant but de-regulated effector protein secretion was still apparent when the 11 aa carboxy-terminal SepL truncates (or any of the other truncates) were transformed into this background (Fig. 4C). This result was also confirmed in the *sepL* frameshift mutant background (Fig. 4D).

Tir binds to the carboxy terminus of SepL

To determine if any of the effector proteins directly interact with SepL, the secreted supernatant effector proteins from a *sepL* mutant were separated by SDS-PAGE and a Far-Western was carried out to examine their interaction with 6× His-tagged SepL. Using this approach, SepL only interacted clearly with one protein in the bacterial supernatant and this protein was of a molecular weight equivalent to Tir (Fig. 5A). This interaction was confirmed using a GST–SepL construct that bound to 6× His-tagged Tir (Fig. 5B). As a control, His-tagged Tir bound to GST–CesT (and GST–SepL) but not to GST alone. As an 11 aa deletion at the carboxy terminus of SepL failed to limit Tir secretion, we hypothesized that the SepL interaction with Tir that limits effector protein secretion requires the carboxy terminus of SepL. This was confirmed as 6× His-tagged Tir was shown to bind to full-length SepL–eGFP but not to any of the SepL truncates (Fig. 5C). We next wanted to determine if the carboxy terminus of SepL alone could bind to Tir. The final 48 aa of SepL was fused to GST and immobilized on a glutathione-sepharose 4B column. This region was selected based on a comparative domain analysis with YopN/TyeA (Fig. 8). Tir-His was able to bind to this hybrid protein but not GST alone (Fig. 5D).

The Far-Western analysis indicated that only Tir was interacting with SepL from the different secreted effector proteins. However, this may be a result of conformation or levels of the proteins present in this type of analysis. To determine if SepL could interact with another hyper-secreted effector protein, NleA was expressed with either an amino- or carboxy-terminal 6× His-tag but neither construct demonstrated any interaction with immobilized GST–SepL (Fig. S1). Therefore, despite the fact that SepL regulates the secretion of a combination of effector proteins, the data presented here indicate that the interaction of SepL with effector proteins may be limited to Tir and this interaction is prevented by removal of the final 11 aa of SepL and that the final 48 aa of SepL are sufficient to bind to Tir.

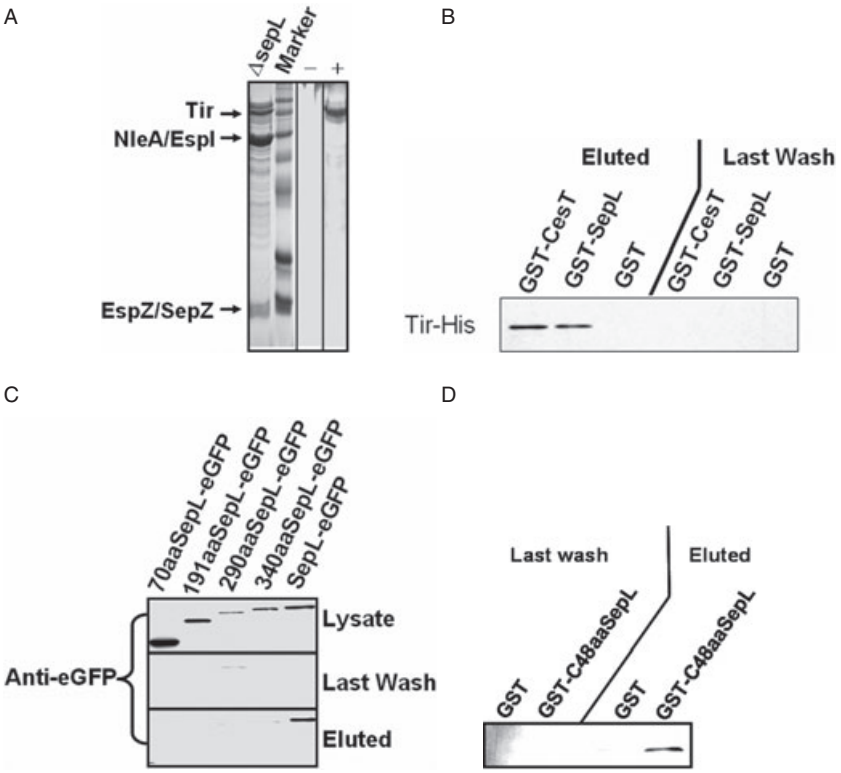

Fig. 5. SepL binds to Tir.
A. Detection of supernatant proteins that bind to SepL. Supernatant proteins from a *sepL* mutant (ZAP1143) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with SepL-His (+) prepared from *E. coli* BL21. As a control (–), the incubation with SepL-His was omitted. The Far-Western was developed following incubation with an anti-penta-His antibody as described in *Experimental procedures*.
B. Detection of Tir binding by immobilized SepL. His-tagged Tir was prepared in *E. coli* BL21 and incubated with immobilized GST–SepL, GST–CesT and GST alone. Following elution, Tir-His was detected by Western blotting.
C. The C-terminus of SepL is required for Tir binding. 6× His-tagged Tir was purified on nickel-NTA columns and *E. coli* K-12 (AAEC185) lysates, containing the different indicated truncates of SepL fused to eGFP, were run through the columns. Following washes, proteins were eluted and separated by PAGE. SepL constructs were then detected by Western blotting using an anti-GFP antibody.
D. The carboxy terminus of SepL is sufficient to bind to Tir. The C-terminal 48 aa of SepL was fused to GST and immobilized onto a column. 6× His-tagged Tir bound to the 48 aa C-terminal SepL construct and was detected in the eluate by Western blotting.

Analysis of Tir domains that interact with SepL and CesT

Tir is stabilized by CesT that is also required for efficient secretion of Tir (Abe *et al*., 1999; Elliott *et al*., 1999). CesT is also the main chaperone for the other effector proteins that are hyper-secreted in a *sepL* mutant (Thomas *et al*., 2005). Prevention of Tir secretion may simply require SepL binding to Tir but the impact of CesT in this interaction is unknown. To determine where SepL and CesT bind to Tir, different Tir constructs were expressed as His-tagged fusions and interactions with immobilized GST–SepL, GST–CesT and GST alone were analysed (Fig. 6). Previous research has shown that the N-terminal 233 aa of Tir contains a CesT binding domain (Abe *et al*., 1999; Elliott *et al*., 1999). Our results confirmed that the first 200 aa of Tir bound to CesT (Fig. 6). However, another CesT binding domain was mapped in Tir deleted for its first 200 aa. Even deletion of the first 382 aa of Tir still produced a polypeptide that could bind to CesT. By contrast, the first 200 aa of Tir did not interact with SepL but the remainder of the protein did bind to SepL as did the 382 amino-terminal truncate. The data indicate that there are at least two regions in Tir that can interact with CesT and that one of these could compete with SepL–Tir binding.

The interaction of Tir with SepL controls the timing of secretion

As an 11 aa deletion of SepL retains the capacity to export translocon proteins but is unable to limit effector protein secretion, it raised the possibility that effector protein export was now occurring at the same time as translocon export as the capacity of SepL to bind Tir potentially sequesters Tir export and somehow limits the secretion of other effector proteins during translocon assembly. To test this, the timing of EspD and Tir secretion was analysed in

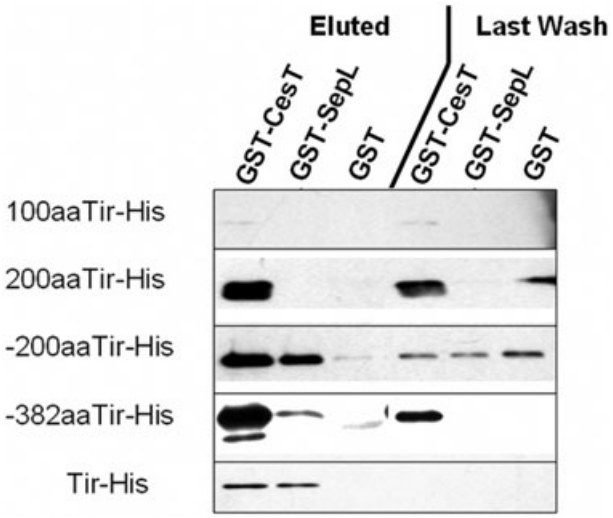

Fig. 6. SepL and CesT binding domains of Tir. Different 6× His-tagged truncates of Tir were incubated with immobilized GST–CesT, GST–SepL and GST alone. Following elution, 6× His-tagged Tir constructs were detected as described in the *Experimental procedures*. 100aaTir-His and 200aaTir-His are His-tagged constructs containing the first 100 and 200 aa of Tir respectively. The first 200 aa of Tir is known to contain a CesT binding region (Abe *et al*., 1999; Elliott *et al*., 1999). –200aaTir-His and –382aaTir-His are His-tagged constructs containing Tir without the first 200 and 382 aa respectively.

the wild-type strain and a *sepL* mutant complemented with either full-length *sepL* or the 11 aa carboxy-terminal deletion. Bacteria were cultured initially in a medium (Luria–Bertani, LB) that is not permissive for T3S and then transferred to a medium (MEM-HEPES) that induces T3S. Following the transition, samples were taken at regular intervals and the levels of secreted Tir and EspD determined as described in *Experimental procedures*. For the full-length SepL complement in the *sepL* deletion, EspD secretion was detectable but not Tir at early time points (Fig. 7A). This pattern was similar in the wild-type strain (Fig. 7A) By contrast, in the *sepL* mutant complemented with the C-terminal 11 aa deletion of SepL, Tir secretion was detectable along with EspD secretion at early time points (Fig. 7A) following the induction of T3S. While it is appreciated that Tir secretion levels are higher in the truncate-complemented background it is clear from analysis of the EspD/Tir secretion ratios (Fig. 7B) that Tir secretion is no longer delayed in the *sepL* mutant complemented with the 11 aa truncate by comparison with full-length *sepL* complementation or the wild type. Consequently, secretion hierarchy is disrupted when the capacity of SepL to bind Tir is removed.

Discussion

SepL and SepD are critical proteins controlling the switch between translocon and effector protein secretion in A/E *E. coli*. From the current study, we propose that SepL directly contributes to the T3S hierarchy by binding to Tir and through this sequestration prevents the secretion of Tir and other effector proteins while the translocon components are exported. This activity requires the carboxy terminus of SepL and can be separated from other phenotypes associated with SepL, including its membrane localization, SepD binding and translocon export.

The C-terminus of SepL shares some homology with TyeA and the remainder of SepL some homology with YopN, both from *Yersinia* spp. (Pallen *et al*., 2005) (Fig. 8). TyeA controls the export of specific effector proteins so it was logical to investigate C-terminus deletions

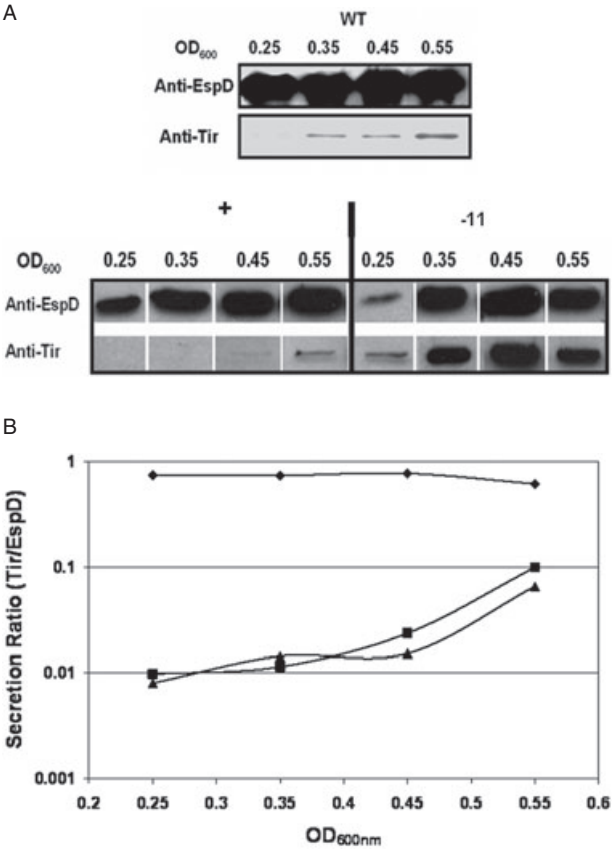

Fig. 7. Secretion timing is altered by deletion of the carboxy terminus of SepL.
A. *E. coli* O157:H7 (ZAP193) (top panel) and the *sepL* deletion (ZAP1143) (second panel) complemented by either full-length SepL (pDW6) or SepL with a deletion of the final 11 aa (pDW30) were cultured in LB that represses T3S and then transferred into MEM-HEPES that induces T3S.
B. Samples were taken at defined optical densities and the levels of secreted EspD and Tir determined as described in *Experimental procedures*. The cultures were repeated in triplicate and the blots shown represent the secretion patterns from one set from which the ratio of secreted Tir to EspD is also shown. Wild type, ZAP193 (▲); Δ*sepL*, ZAP1143 complemented with full-length SepL (■), or with the C-terminal –11 aa truncate (◆).

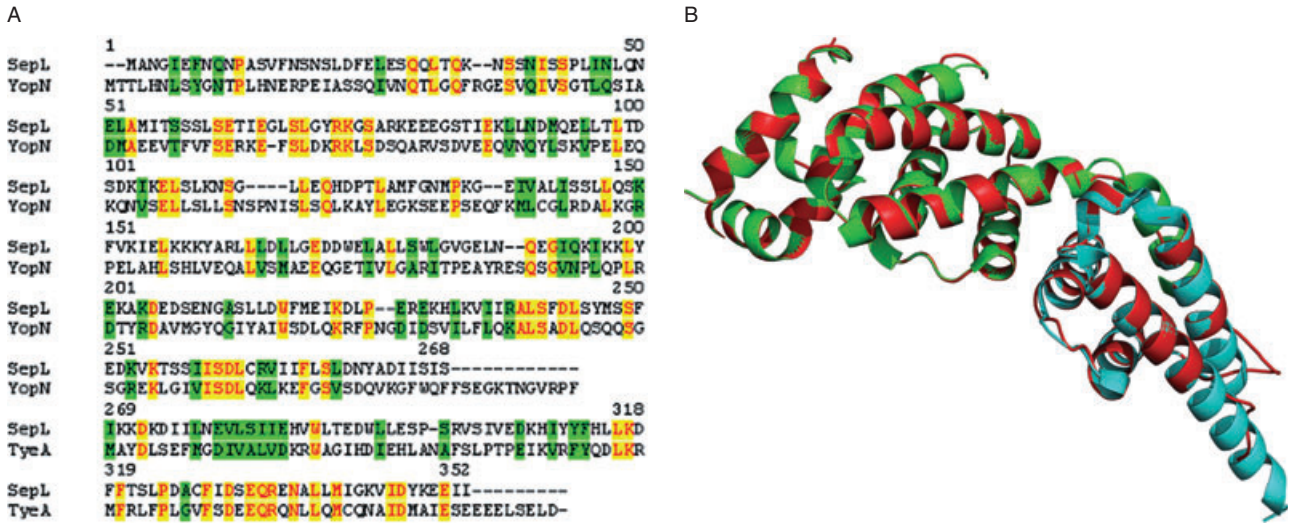

Fig. 8. Protein sequence and predicted structural comparisons between SepL and YopN/TyeA.
A. Amino acid alignment of SepL (gb|AAG58821) with YopN (gb|AAS58563) and TyeA (gb|AAS58564) of *Yersinia pestis*. Align X software (Invitrogen) was used to align the first 268 aa of SepL with YopN (294 aa) and the remaining 84 aa of SepL with TyeA (93 aa). Identical aa are shown by red text on a yellow background; blocks of similar amino acids are highlighted in green.
B. Structure of the YopN76-293–TyeA complex overlaid with SepL (red). YopN and TyeA are depicted in green and cyan respectively. The alignment and mapping was performed using Swissmodel using the 1XL3 file from Schubot *et al*. (2005) as a model. PyMol (http://pymol.sourceforge.net/) was used to generate the figure.

of SepL to see if these can separate the known activities of SepL and this did prove to be the case. Membrane localization was investigated by both imaging and biochemical analysis of SepL–eGFP constructs. While some cleavage of eGFP was detected from these heterologous proteins it was evident that only the full-length SepL and SepL constructs with deletions of either 11 aa or 61 aa from the C-terminus were able to localize to membrane-containing fractions. The same three SepL constructs were also able to bind to SepD *in vitro*. In addition, a *sepD* mutation reduced the levels of SepL–eGFP associated with the membrane-containing fractions and led to its asymmetric distribution in the bacterial cell. SepD is expressed from LEE2 along with T3S basal apparatus proteins and it is likely that the SepL interaction with SepD is responsible for the membrane localization of SepL, possibly to the T3S apparatus, although higher-resolution imaging is required to investigate this. This work confirms previous research that indicated that SepL can be membrane-associated (Kresse *et al*., 2000; Deng *et al*., 2005), although one report for EPEC concluded that it was only cytoplasmic (O'Connell *et al*., 2004). This same study also concluded that effector proteins were not hyper-secreted in a *sepL* mutant, although this does not agree with the current study and other published research (Kresse *et al*., 2000; Deng *et al*., 2004; 2005). In our work the ratios obtained for the SepL–eGFP fusion in the different fractions were variable, potentially indicating only a weak association of SepL with the membrane, potentially via SepD. Each membrane preparation may disassociate this interaction to different levels and account for the variation seen.

SepL-dependent translocator secretion was abolished with a C-terminus deletion of 61 aa, even though this construct could still bind SepD and localize to the membrane. By contrast, only the full-length SepL protein was able to restore normal levels of Tir and other effector protein secretion in a *sepL* mutant. Therefore, the 11 aa deletion still functioned to some extent to export translocon proteins but had lost effector protein secretion control. As the final 11 aa of SepL were essential for controlling effector protein secretion, we examined whether any of these hyper-secreted proteins could actually bind to SepL. Tir was shown to interact with SepL and this interaction required the carboxy-terminal 11 aa of SepL. In addition, the C-terminal 48 aa of SepL, when combined with GST, was capable of binding to Tir (Fig. 5D). When the structure of the YopN76-293–TyeA complex (Schubot *et al*., 2005) is overlaid with SepL, it is evident that the final 48 aa of SepL map to the final two alpha helices of TyeA and this domain is sufficient to interact with Tir. The sequence divergence at the C-terminal 12 aa between TyeA and SepL may relate to the recognition of different proteins in the two organisms. Our work also demonstrates that the region of SepL required for SepD binding must lie within the YopN homologous region (Fig. 8). There was no evidence from our work that any of the other secreted effector proteins could bind directly to

SepL, although this cannot be discounted as they have not all been tested individually. The Far-Western analysis only identified one clear binding partner, Tir. While NleA is known to be secreted at higher levels in a *sepL* mutant, it did not bind to SepL *in vitro* using similar approaches that were successful with Tir. Therefore, it appears likely that the Tir–SepL interaction is critical in limiting the secretion of effector proteins in general. This finding fits well with recently published research (Thomas *et al*., 2007) that demonstrated that Tir is required for hyper-secretion of other effector proteins in a *sepD* mutant background.

Our previous work has demonstrated that LEE4 and LEE5 are co-ordinately expressed, indicating that Tir will be produced in individual bacteria while the translocon is being assembled (Roe *et al*., 2004). Therefore, we investigated the hypothesis that the binding of Tir by SepL actually sequesters it and prevents its early release while the translocon is being assembled. The timing of release of Tir and the translocon protein EspD were investigated using a shift in culture conditions from a non-permissive to a permissive medium for T3S. Under these conditions, Tir secretion was demonstrated to be delayed in the wild type and a *sepL* deletion strain complemented by full-length SepL. However, Tir secretion occurred at the same time as EspD secretion when the strain was complemented with SepL deleted for the C-terminal 11 aa. The data support the proposition that the timing of Tir and effector protein secretion is directly controlled by SepL binding to Tir. The altered timing may also account for the higher levels of secreted effector proteins found in bacterial supernatants of *sepL* mutants as these can be exported over a longer period by each cell. However, as the deletion of the C-terminus of SepL will have other effects on SepL function, we cannot rule out that another mechanism may be responsible for limiting effector protein export.

How Tir binding to SepL could prevent secretion of other effector proteins is not understood but it must presumably stall a series of T3S apparatus interactions with effector proteins prior to EscN/ATPase-driven export. Another key question is how such a mechanism is then switched once translocon export has finished, allowing effector protein export. It has been suggested that opening a conduit to the host cell via the translocon could induce a change in local ion concentrations in particular calcium, which may disassociate or alter the SepL/SepD complex (Deng *et al*., 2005). Our current work has indicated that the SepL–Tir interaction could also be a target for such a trigger. Alternatively, SepL and/or SepD may have limited stability so their activity is only for a defined period. Another possibility is that SepL (like YopN) may be secreted to initiate effector secretion (Pallen *et al*., 2005). We have tested whether His-tagged fusions to SepL or the SepL region homologous to YopN (the first 267 aa of SepL) are exported into the bacterial supernatant. There was no evidence for this even though both were expressed inside the bacterial cell (data not shown). An indication of a potential mechanism to release Tir from SepL comes from previous research that has shown a direct interaction between Tir and EscD (Pas) (Kresse *et al*., 1998). EscD is considered to be a protein in the inner membrane complex of the T3S (Ogino *et al*., 2006) and when deleted prevents both translocon and effector protein secretion. However, plasmid complementation of *escD* led to high levels of Tir secretion (Ogino *et al*., 2006), indicating that overexpression of EscD also leads to loss of Tir secretion control. We have shown that SepL binds directly to EscD (D. Wang and D. Gally, unpubl. data) and this binding requires the same final 11 aa of SepL that are required for SepL binding to Tir. It may be that two different organizations of the SepL–SepD complex are required to permit translocator and effector protein export with different binding partners at the C-terminus of SepL.

Normal levels of Tir secretion also require presentation by its chaperone, CesT, and will require the interaction of complexed Tir with the ATPase, EscN, which then energizes the export of Tir (Gauthier and Finlay, 2003). Many other effector proteins utilize CesT, especially those known to be hypersecreted in a *sepL* mutant (Thomas *et al*., 2005). Our *in vitro* data indicate that the CesT binding domain shown in the amino-terminal third of Tir is not the only region of Tir that interacts with CesT. It remains possible that while the amino-terminal domain is important for stability a further domain is necessary for its export. Multiple chaperone binding sites in Tir have been suggested by others (Elliott *et al*., 1999). In the context of the current work, SepL and CesT were shown to bind in the C-terminal half of Tir and preliminary data support competitive interactions between SepL and CesT for Tir binding. CesT and Tir are both expressed from the LEE5 transcript (Elliot *et al*., 1999) and LEE4 and LEE5 are co-ordinately expressed (Roe *et al*., 2004). As a consequence, Tir stabilized by CesT will be present in the cell while EspA filaments are assembled. CesT–Tir must interact with the T3S system to allow Tir release from CesT and its subsequent export. Therefore, we propose that the CesT–Tir complex interacts with a SepD–SepL 'filter' but the interaction of Tir with SepL becomes the stalling point for effector protein secretion. Once Tir export is triggered by disassociation of this interaction, other effectors can then be exported but these also have to pass through the SepD–SepL 'filter', perhaps requiring an interaction of CesT and/or effectors with SepD. This combination of possible interactions is the focus of ongoing research.

Table 1. Plasmids used in the study.

Plasmid	Description
pACYC184	Low-copy-number cloning vector
pGEX-4T-2	Plasmid contained GST gene fusion system from Amersham Biosciences
pET21d	Plasmid contained 6× His gene fusion system from Novagen
pTir-His	pET21d digested with XbaI/XhoI; fragment contained full-length *tir* gene amplified from ZAP193 and inserted
p100Tir-His	pET21d digested with XbaI/XhoI; fragment contained the first 100 aa *tir* gene amplified from ZAP193 and inserted
p200Tir-His	pET21d digested with XbaI/XhoI; fragment contained the first 200 aa *tir* gene amplified from ZAP193 and inserted
p-200Tir-His	pET28a digested with NdeI/XhoI; fragment contained the 201–558 aa *tir* gene amplified from ZAP193 and inserted
p-382Tir-His	pET28a digested with NdeI/XhoI; fragment contained the 382–558 aa *tir* gene amplified from ZAP193 and inserted
pDG028	Low-copy-number vector containing *sacB/kan* cassette, laboratory stock
pIB307	pMAK705-based vector for allelic exchange; temperature-sensitive replicon (Blomfield *et al.*, 1991)
pAJR70	pACYC184 digested with BamHI; *egfp* gene cloned BamHI/BglII (Roe *et al.*, 2003)
pDW6	pAJR70 digested with BamHI/KpnI; *sepL* with its own promoter amplified from ZAP193, cloned in frame 5′ to *egfp*
pDW7	pIB307 digested with XbaI/HindIII; fragment contained 1165 bp *sepL* downstream sequence amplified from ZAP193 and inserted
pDW8	pDW7 digested with KpnI/BamHI; fragment contained 985 bp *sepL* upstream sequence amplified from ZAP193 and inserted
pDW9	pGEX-4T-2 digested with BamHI/SmaI; fragment contained full-length *sepL* gene amplified from ZAP193 and inserted
pDW10	pIB307 cut with KpnI/BamHI; fragment contained 985 bp *sepL* upstream sequence amplified from ZAP193 and inserted
pDW11	pDW8 digested with BamHI; *sacB/kan* cassette inserted
pDW15	pGEX-4T-2 digested with BamHI/SmaI; fragment contained full-length *sepD* gene amplified from ZAP193 and inserted
pDW20	pAJR70 digested with BamHI/KpnI; fragment contained full-length *sepD* gene amplified from ZAP193, cloned in frame 5′ to *egfp*
pDW21	pDW307 digested with BamHI/SacI; fragment contained 746 bp *sepD* upstream sequence amplified from ZAP193 and inserted
pDW22	pDW21 digested with BamHI/AseI; fragment contained 805 bp *sepD* downstream sequence amplified from ZAP193 and inserted
PDW23	pDW22 digested with BamHI; *sacB/kan* cassette inserted
pDW24	pACYC184 digested with BamHI; full-length *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW26	pACYC184 digested with BamHI/KpnI; 1–51 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW27	pACYC184 digested with BamHI/KpnI; 1–210 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW28	pACYC184 digested with BamHI/KpnI; 1–573 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW29	pACYC184 digested with BamHI/KpnI; 1–870 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW30	pACYC184 digested with BamHI/KpnI; 1–1020 bp *sepL* gene with its own promoter amplified from ZAP193 and inserted
pDW40	pGEX-4T-2 digested with BamHI/SmaI; fragment contained full-length *cesT* gene amplified from ZAP193 and inserted
pDW42	pET21d digested with XbaI/XhoI; fragment contained full-length *sepL* gene amplified from ZAP193 and inserted
pDW45	pACYC184 digested with BamHI; 1–801 bp *sepL* gene with its own promoter amplified from ZAP193 (6× His-tag was introduced into C-terminus by primer) and inserted
pDW46	pACYC184 digested with BamHI; 1–870 bp *sepL* gene with its own promoter amplified from ZAP193 (6× His-tag was introduced into C-terminus by primer) and inserted
pDW47	pACYC184 digested with BamHI; 1–1020 bp *sepL* gene with its own promoter amplified from ZAP193 (6× His-tag was introduced into C-terminus by primer) and inserted
pDW48	pACYC184 digested with BamHI; full-length *sepL* gene with its own promoter amplified from ZAP193 (6× His-tag was introduced into C-terminus by primer) and inserted
pDW50	pGEX-4T-2 digested with BamHI/SmaI; fragment contained caboxy-terminal 48 aa residue of *sepL* gene amplified from ZAP193 and inserted
pDW51	pIB307 digested with KpnI/XbaI; fragment contained *sepL* gene and flanking regions (upstream 1 kb + downstream 1 kb) amplified from ZAP193 and inserted
pDW52	pDW51 was site-direct mutated by inserting a base pair at 345 bp of *sepL orf*

Logically, secretion of Tir and effector proteins should be restricted until the translocon is assembled so they can be secreted directly into the host cell. If Tir is secreted prior to this it may interfere with translocon assembly and perhaps, more importantly, it may bind to surface-expressed intimin and therefore block the subsequent interaction of intimin with host membrane-inserted Tir. A/E *E. coli* along with *Citrobacter rodentium* are unique in having SepL and SepD proteins and are the only bacterial pathogens to date known to inject their own receptor. This may not be a coincidence as tight control over the release of this receptor is required.

Experimental procedures

Bacterial strains, plasmids, oligonucleotides, media and antibodies

The bacterial strains, media, antibodies and plasmids used in the study are described in Tables 1 and 2. Table 3 lists the oligonucleotide primers used. MEM-HEPES is minimal essential medium with HEPES buffer (Sigma), containing additional glucose to a final concentration of 0.2%. LB broth was also used (Oxoid). Antibiotics were included when required at the following concentrations: chloramphenicol 12.5 μg ml^{-1}, kanamycin 25 μg ml^{-1} and ampicillin 50 μg ml^{-1}.

Table 2. Bacterial strains and antibodies.

Strains	Details
ZAP193	*E. coli* O157:H7 *stx*-, NCTC 12900
ZAP198	*E. coli* O157:H7 (Naylor *et al*., 2003)
ZAP1004	*E. coli* O157 *stx* Nal^r Δ*ler* (Low *et al*., 2006)
ZAP1143	*E. coli* NCTC 12900; O157 *stx* Nal^r Δ*sepL* (total deletion)
ZAP1144	*E. coli* NCTC 12900; O157 *stx* Nal^r Δ*sepD* (total deletion)
ZAP1211	*E. coli* NCTC 12900; O157 *stx* Nal^r Δ*sepL* (frameshift mutation by insertion of an additional base at nucleotide position 345 of *sepL*)
Media	Details
LB	LB broth, Oxoid
M9	M9 minimal medium was modified with a final glycerol concentration of 0.4%, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, MEM non-essential amino acids solution (Sigma), MEM amino acids solution (Sigma)
MEM-HEPES	Minimal essential medium with HEPES buffer (Sigma), glucose was added to MEM-HEPES to give a final concentration of 0.2%
Note	Antibiotics were included when required at the following concentrations: chloramphenicol 12.5 μg ml^{-1}, kanamycin 25 μg ml^{-1}
Antibodies	Details
Anti-GFP	Mouse monoclonal (Clontech)
Anti-EspD	Mouse monoclonal (gift from Prof. Trinad Chakraborty)
Anti-Tir	Mouse monoclonal (gift from Prof. Trinad Chakraborty)
Anti-OmpA	Rabbit polyclonal (gift from Prof. John Leong)
Anti-GroEL	Rabbit polyclonal (Stressgen)
Anti-His	Mouse Penta-His antibody (Qiagen)
Anti-Rabbit Igs	Peroxidase-conjugated Swine anti-rabbit immunoglobulins, mainly IgG, HRP, DAKO
Anti-Mouse Igs	Polyclonal goat anti-mouse immunoglobulins, mainly IgG, HRP, DAKO
Anti-Rabbit Igs, FITC/TRITC conjugated	Goat anti-rabbit IgG, FITC/TRITC, DAKO

Preparation of secreted proteins and bacterial fractions for protein analyses

Bacteria were cultured in 50 ml of MEM-HEPES at 37°C (200 r.p.m.) to an OD_{600} of 0.8 unless specifically stated. The bacterial cells were pelleted by centrifugation at 4000 *g* for 20 min, and supernatants were passed through filters (0.45 μm). The proteins were precipitated overnight with 10% TCA, and separated by centrifugation at 4000 *g* for 30 min (4°C); the proteins were suspended in 150 μl of 1.5 M Tris (pH 8.8). The bacterial pellet was initially suspended in 150 μl of sonication buffer [10 mM Tris-HCl (pH 7.5), 0.5 mM PMSF, aprotinin (0.5 μg ml^{-1})] and sonicated on ice. Cell envelopes and unbroken bacteria were removed by two rounds of centrifugation (5000 *g* for 10 min at 4°C). The supernatant (whole-cell fraction) was removed and the membranes pelleted by ultra-centrifugation of the samples for 1 h at 500 000 *g* at 4°C. The supernatant containing cytoplasmic proteins was collected. The membrane preparation was washed twice with sonication buffer and re-suspended in 150 μl of SDS sample buffer. Proteins were separated by SDS-PAGE using standard methods and Western blotting performed as described previously (Roe *et al*., 2003; Naylor *et al*., 2005) using the relevant antibodies listed in Table 2. Tir and EspD secretion levels were measured following enhanced chemi-luminescence detection from Western blots using Multi-analyst (Bio-Rad) software.

SepL analysis

Full-length SepL and different carboxy-terminal truncates of SepL (Fig. 1) were fused to eGFP in pAJR70 (Roe *et al*., 2003) using the primers described in Table 3. A 6× His-tag was introduced at the carboxy end of SepL and three of the truncated SepL proteins (267, 290 and 340 aa) by PCR before cloning into pACYC184 (Table 1). All constructs were confirmed by sequencing.

Construction of GST and 6× His-tagged proteins and binding assays

For the GST–SepL construct, *sepL* was amplified from EHEC O157 ZAP193 by PCR using the primers sepL 5′g and sepL 3′g. The resulting PCR product was digested with BamHI and SmaI, and cloned into the BamHI and SmaI sites of pGEX-4T-2. This creates a GST–SepL hybrid protein fusion (in pDW9) used in GST pull-down assays. A similar stategy was used to clone the 48 aa carboxy terminus of SepL, SepD and CesT using the primers described in Table 3. For His-tagged proteins, *tir* and *sepD* open reading frames were amplified by the primers listed in Table 3, digested with XbaI and XhoI and cloned into the XbaI and XhoI sites of pET21d. For *sepL* and *tir* domain analyses, the amplified fragments were cloned into pET28a via NdeI and XhoI to create N-terminal 6× His-tags. All constructs were expressed in *E. coli* BL21 (Table 2) following IPTG induction (0.1 mM). The GST fusions were expressed in AAEC 185 (an *E. coli* K-12 derivative, Table 2) and the His-tag fusions in *E. coli* BL21, both following IPTG induction in LB (0.1 mM) at $OD_{600} = 0.5$. For protein preparations, the bacteria were harvested at 4000 r.p.m. (4°C) for 30 min. 2 h post IPTG inoculation. The bacterial pellet was suspended in PBS and sonicated. The supernatant was collected by centrifugation at 12 000 *g* for 10 min at 4°C. For the GST fusions, the supernatant was

Table 3. Oligonucleotides used in the study. The underlined letters highlight the restriction sites used in the relevant primers.

Primer name	Application	Sequence
sepLfull-5′	pDW6,24,26,27,28,29,30,45,46,47,48	CGGGATCCATGGCTAATGGTATTGAATTTAATCTTACCAGATGCTTGCTTTATTG
sepLfull-3′	pDW6	GGGGTACCAATAATTTCCTCCTTATAGTC
sepL 5′*lhs*	pDW8, pDW10	CGGGGTACCTTTTTAAACTCTGATGCCAG
sepL 3′*lhs*	pDW8, pDW10	CGCGGATCCTGGAAACTCACGTAATC
sepL 5′*rhs*	pDW7	TGCTCTAGATATTAATTACTCAATAATTTTTTTG
sepL 3′*rhs*	pDW7	CCAAGCTTAACAATTTTACTTTTTTGTG
sepL 5′g	pDW9	CGGGATCCATGGCTAATGGTATTGAATTTAATC
C48aasepL 5′g	pDW50	CGGGATCCGAAGATAAACATATTTATTATTTTC
sepL 3′g	pDW9,50	CAACCCGGGTCAAATAATTTCCTCCTTATAGTC
sepL 3BamH	pDW24	CGGGATCCTCAAATAATTTCCTCCTTATAGTC
sepD 5′*lhs*	pDW21	GCGAGCTCCAGCGATCTCAGTTTCGATG
sepD 3′*lhs*	pDW21	GCCGGATCCCATACATATTACCCGTCCTG
sepD 5′*rhs*	pDW22	GCGGATCCCCGCCAACACACTTGTTTTC
sepD 3′*rhs*	pDW22	GCATTAATCGGTCTTTTACAACAACTGC
sepD 5′g	pDW15	CGGGATCCATGAACAATAATAATGGCATAG
sepD 3′g	pDW15	CCCCGGGTTACACAATTCGTCCTATATCAG
sepD 5′	pDW20	CGGGATCCCTAAAGAAAGAGAAAAATGCG
sepD 3′	pDW20	GGGGTACCTTACACAATTCGTCCTATATCAG
cesT 5′g	pDW40	CGGGATCCATGTCATCAAGATCTGAACTTTTA
cesT 3′g	pDW40	CCCCGGGTTATCTTCCGGCGTAATAATGTTTA
sepL51-3′	pDW26	GGGGTACCAGAATTAAAAACAGATGCGGGG
sepL210-3′	pDW27	GGGGTACCACCTTTGCGATATCCCAGGC
sepL573-3′	pDW28	GGGGTACCAGCCTTTTCATAAAGCTTCTTG
sepL870-3′	pDW29	GGGGTACCTGTTAGCCAGACATGTTCAATA
sepL1020-3′	pDW30	GGGGTACCAATCATTAATAATGCATTCTCTC
sepL801-h3	pDW45	CGGGATCCTCAGTGGTGGTGGTGGTGGTGGATCGAAATAATATCTGCATAGT
sepL870-h3	pDW46	CGGGATCCTCAGTGGTGGTGGTGGTGGTGTGTTAGCCAGACATGTTCAATA
sepL1020-h3	pDW47	CGGGATCCTCAGTGGTGGTGGTGGTGGTGAATCATTAATAATGCATTCTCTCT
sepLFL-h3	pDW48	CGGGATCCTCAGTGGTGGTGGTGGTGGTGAATAATTTCCTCCTTATAGTCGA
tir5h	pTir-His	AAAAATCTAGAAAAGGAGATATTTATGCCTATTG
tir3h	pTir-His	AAAAACTCGAGGACGAAACGATGGGATCCCGGCG
100tir3h	p100Tir-His	CCGCTCGAGGTTAAGAGTATCGAGCGGAC
200tir3h	p200Tir-His	CCGCTCGAGAACGCCTTTTGACTCCCCAG
–200tir5h	p-200Tir-His	GGAATTCCATATGGGGGAGTTGAGGGAGTC
–382tir5h	p-382Tir-His	GGAATTCCATATGCTTCATCGAAAAAATCAGCCG
sepL5h	pDW42	GCTCTAGATGGAATATTCATAATTAATGATTAC
sepL3h	pDW42	CCGCTCGAGTCAAATAATTTCCTCCTTATAGTC
sepL5′*allf*	pDW51	GGGGTACCGACATCATTAATTGCGGATATG
sepL3′*allf*	pDW51	GCTCTAGAGACGAGTTCATGGATTTAACC
sepLfs5	pDW52	GCGGGCTGCTGGAACAACACGACTCCTACTTTGGCGATGTTTGGC
sepLfs3	pDW52	GCCAAACATCGCCAAAGTAGGAGTCGTGTTGTTCCAGCAGCCCGC

mixed with PBS-balanced glutathione-sepharose 4B beads for 30 min at room temperature. The beads were separated by centrifugation at 500 *g* for 5 min. An aliquot of the supernatant was saved for analysis and the rest of the supernatant discarded. The beads were washed three times using 10 vols of PBS and separation by centrifugation at 500 *g* for 5 min. The beads were mixed gently in the same volume of Glutathione Elution Buffer (0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl, pH 8.0) and incubated at room temperature for 10 min. Supernatant was collected by centrifugation at 500 *g* for 5 min. The elution and centrifugation step was repeated and the two eluates pooled. Equal volumes of washes and eluates were loaded onto the protein gels. To check initial loading of columns, some eluates were stained with colloidal blue and/or Western blotted to confirm the presence of the expected GST fusion protein and His- or GFP-tagged binding partners.

For His-tagged proteins, these were expressed and purified as above except a Ni-NTA column was used and elution was with the supplied Qiagen buffer. For the *in vitro* binding assays, these were carried out on either glutathione or Ni-NTA columns on which the bait protein was first retained and then the protein being investigated was run through the column in a lysate prepared as above. Following washes, elution was carried out as described above. Eluted samples were analysed by SDS-PAGE followed by colloidal blue staining and Western Blotting.

Construction of sepL *and* sepD *mutants*

The experiments were carried out essentially as described previously (Roe *et al.*, 2003; Emmerson *et al.*, 2006) using allelic exchange methodology. The respective primer sets

used to amplify the *sepL*, *sepD* are described in Table 3, the *ler* deletion was published previously (Low *et al.*, 2006). To generate plasmids for the *sepL* frameshift mutation, a fragment containing *sepL* gene and flanking regions (upstream 1 kb + downstream 1 kb) was amplified from ZAP193 by PCR using sepL 5′*allf* and sepL 3′*allf*. It was digested with by KpnI and XbaI, and cloned into the KpnI and XbaI sites of pIB307. Following the methodology in the Stratagene 'Site-Directed Mutagensis' kit, a single base pair was inserted at 345 bp of *sepL* to generate a plasmid (pDW52) for allelic exchange. Final plasmid constructs (Table 1) were sequenced prior to the deletion exchange and each deletion confirmed by PCR analysis. The *sepL* and *sepD* mutants could be functionally complemented by pDW24 (*sepL*), pDW48 (*sepL*::*6xhis*), pDW6 (*sepL::egfp*) and pDW20 (*sepD::egfp*) (Table 1) to restore translocon (EspD) secretion as determined by Western blotting (Fig. 4A, C and D and data not shown).

Fluorescence imaging

Fluorescence imaging was carried out using a Leica DM LB2 microscope and a 100× objective lens. Narrow-bandwidth filters to excite and detect eGFP/FITC were used (41017 Endow GFP, CHROMA). Images were captured using a Hamamatsu ORCA-ER black and white CCD digital camera. Images were analysed using OpenLab software (Improvision). To measure levels of fluorescence in individual cells, transects were marked on bacteria and the fluorescence levels determined using QFluor software (Leica).

Far-Western analysis

Supernatant proteins were prepared from ZAP1143 (Δ*sepL*) as described in the relevant section above. The secreted proteins were separated by SDS-PAGE using standard methods and then transfered to an enhanced chemiluminescence Nitrocellulose membrane. The membrane was first blocked with 8% milk PBS overnight at 4°C and washed three times with PBS-Tween (0.5% v/v) before being incubated overnight with SepL-His in an *E. coli* BL21 lysate at 4°C. After the incubation, the nitrocellulose membrane was washed three times with PBS-Tween and the standard Western procedure for detecting the 6× His-tags was carried out.

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References

Abe, A., De Grado, M., Pfueetzner, R.A., Sanchez-SanMartin, C., DeVinney, R., Puente, J.L., *et al.* (1999) Enteropathogenic *Escherichia coli* translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. *Mol Microbiol* **33:** 1162–1175.

Blomfield, I.C., Vaughn, V., Rest, R.F., and Eisenstein, B.I. (1991) Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. *Mol Microbiol* **5:** 1447–1457.

Campellone, K.G., Robbins, D., and Leong, J.M. (2004) EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev Cell* **7:** 217–228.

Cheng, L.W., and Schneewind, O. (2000) *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol* **182:** 3183–3190.

Creasey, E.A., Delahay, R.M., Daniell, S.J., and Frankel, G. (2003) Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic *Escherichia coli*. *Microbiology* **149:** 2093–2106.

Daniell, S.J., Delahay, R.M., Shaw, R.K., Hartland, E.L., Pallen, M.J., Booy, F., *et al.* (2001) Coiled-coil domain of enteropathogenic *Escherichia coli* type III secreted protein EspD is involved in EspA filament-mediated cell attachment and hemolysis. *Infect Immun* **69:** 4055–4064.

Day, J.B., Ferracci, F., and Plano, G.V. (2003) Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN*, *tyeA*, *sycN*, *yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phospho-specific antibodies. *Mol Microbiol* **47:** 807–823.

Deibel, C., Kramer, S., Chakraborty, T., and Ebel, F. (1998) EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Mol Microbiol* **28:** 463–474.

Deng, W., Puente, J.L., Gruenheid, S., Li, Y., Vallance, B.A., Vazquez, A., *et al.* (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc Natl Acad Sci USA* **101:** 3597–3602.

Deng, W.Y., Li, Y.L., Hardwidge, P.R., Frey, E.A., Pfuetzner, R.A., Lee, S., *et al.* (2005) Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. *Infect Immun* **73:** 2135–2146.

Elliott, S.J., Hutcheson, S.W., Dubois, M.S., Mellies, J.L., Wainwright, L.A., Batchelor, M., *et al.* (1999) Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol* **33:** 1176–1189.

Emmerson, J.R., Gally, D.L., and Roe, A.J. (2006) Generation of gene deletions and gene replacements in *Escherichia coli* O157:H7 using a temperature sensitive allelic exchange system. *Biol Proced Online* **8:** 153–162.

Garmendia, J., Phillips, A.D., Carlier, M.F., Chong, Y., Schuller, S., Marches, O., *et al.* (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157: H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol* **6:** 1167–1183.

Garmendia, J., Frankel, G., and Crepin, V.F. (2005) Enteropathogenic and enterohaemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infect Immun* **73:** 2573–2585.

Gauthier, A., and Finlay, B.B. (2003) Translocated intimin receptor and its chaperone interact with ATPase of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *J Bacteriol* **185:** 6747–6755.

Jerse, A.E., Yu, J., Tall, B.D., and Kaper, J.B. (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA* **87:** 7839–7843.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A., and Finlay, B.B. (1997) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91:** 511–520.

Knutton, S., Baldwin, T., Williams, P.H., and McNeish, A.S. (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells – basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* **57:** 1290–1298.

Knutton, S., Rosenshine, I., Pallen, M.J., Nisan, I., Neves, B.C., Bain, C., *et al.* (1998) A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* **17:** 2166–2176.

Kresse, A.U., Schulze, K., Deibel, C., Ebel, F., Rohde, M., Chakraborty, T., and Guzman, C.A. (1998) Pas, a novel protein required for protein secretion and attaching and effacing activities of enterohaemorrhagic *Escherichia coli*. *J Bacteriol* **180:** 4370–4379.

Kresse, A.U., Beltrametti, F., Muller, A., Ebel, F., and Guzman, C.A. (2000) Characterization of SepL of enterohaemorrhagic *Escherichia coli*. *J Bacteriol* **182:** 6490–6498.

Low, A.S., Rosser, T., Holden, N., Roe, A.J., Constantinidou, C., Hobman, J., *et al.* (2006) Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic *Escherichia coli* O157:H7. *Environ Microbiol* **8:** 1033–1047.

McDaniel, T.K., Jarvis, K.G., Donnenberg, M.S., and Kaper, J.B. (1995) A genetic-locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci USA* **92:** 1664–1668.

Naylor, S.W., Low, J.C., Besser, T.E., Mahajan, A., Gunn, G.J., Pearce, M.C., *et al.* (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun* **71:** 1505–1512.

Naylor, S.W., Roe, A.J., Nart, P., Spears, K., Smith, D.G., Low, J.C., and Gally, D.L. (2005) *Escherichia coli* O157:H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology* **151:** 2773–2781.

Neves, B.C., Mundy, R., Petrovska, L., Dougan, G., Knutton, S., and Frankel, G. (2003) CesD2 of enteropathogenic *Escherichia coli* is a second chaperone for the type III secretion translocator protein EspD. *Infect Immun* **71:** 2130–2141.

O'Connell, C.B., Creasey, E.A., Knutton, S., Elliott, S., Crowther, L.J., Luo, W., *et al.* (2004) SepL, a protein required for enteropathogenic *Escherichia coli* type III translocation, interacts with secretion component SepD. *Mol Microbiol* **52:** 1613–1625.

Ogino, T., Ohno, R., Sekiya, K., Kuwae, A., Matsuzawa, T., Nonaka, T., *et al.* (2006) Assembly of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *J Bacteriol* **188:** 2801–2811.

Pallen, M.J., Beatson, S.A., and Bailey, C.M. (2005) Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion. *BMC Microbiol* **5:** 9.

Roe, A.J., Yull, H., Naylor, S.W., Woodward, M.J., Smith, D.G.E., and Gally, D.L. (2003) Heterogeneous surface expression of EspA translocon filaments by *Escherichia coli* O157:H7 is controlled at the posttranscriptional level. *Infect Immun* **71:** 5900–5909.

Roe, A.J., Naylor, S.W., Spears, K.J., Yull, H.M., Dransfield, T.A., Oxford, M., *et al.* (2004) Co-ordinate single-cell expression of LEE4- and LEE5-encoded proteins of *Escherichia coli* O157:H7. *Mol Microbiol* **54:** 337–352.

Schubot, F.D., Jackson, M.W., Penrose, K.J., Cherry, S., Tropea, J.E., Plano, G.V., and Waugh, D.S. (2005) Three-dimensional structure of a macromolecular assembly that regulates type III secretion in *Yersinia pestis*. *J Mol Biol* **346:** 1147–1161.

Sundberg, L., and Forsberg, A. (2003) TyeA of Yersinia pseudotuberculosis is involved in regulation of Yop expression and is required for polarized translocation of Yop effectors. *Cell Microbiol* **5:** 187–202.

Thomas, N.A., Deng, W., Baker, N., Puente, J., and Finlay, B.B. (2007) Hierarchical delivery of an essential host colonization factor in enteropathogenic *Escherichia coli*. *J Biol Chem* **282:** 29634–29645.

Thomas, N.A., Deng, W., Puente, J.L., Frey, E.A., Yip, C.K., Strynadka, N.C., and Finlay, B.B. (2005) CesT is a multi-effector chaperone and recruitment factor required for the efficient type III secretion of both LEE- and non-LEE-encoded effectors of enteropathogenic *Escherichia coli*. *Mol Microbiol* **57:** 1762–1779.

Wachter, C., Beinke, C., Mattes, M., and Schmidt, M.A. (1999) Insertion of EspD into epithelial target cell membranes by infecting enteropathogenic *Escherichia coli*. *Mol Microbiol* **31:** 1695–1707.

Wainwright, L.A., and Kaper, J.B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Mol Microbiol* **27:** 1247–1260.

Yip, C.K., Kimbrough, T.G., Felise, H.B., Vuckovic, M., Thomas, N.A., Pfuetzner, R.A., *et al.* (2005) Structural characterization of the molecular platform for type III secretion system assembly. *Nature* **435:** 702–707.

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